ECOLOGY AND PATHOGENICITY OF THE ANAEROBIC PATHOGEN *CLOSTRIDIUM BOTULINUM* SEEN IN FARMED FISH SHELLFISH AND FISHERY PRODUCTS

THESIS

SUBMITTED TO

CENTRAL INSTITUTE OF FISHERIES EDUCATION

(DEEMED UNIVERSITY)

VERSOVA, MUMBAI

in partial fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

(MARICULTURE)

BY

K.V.LALITHA

POSTGRADUATE PROGRAMME IN MARICULTURE

CENTRAL MARINE FISHERIES RESEARCH INSTITUTE

P.B.NO. 1603, TATAPURAM.P.O. COCHIN-682014

MARCH 1998

DECLARATION

I hereby declare that this thesis entitled 'Ecology and pathogenicity of the anaerobic pathogen *Clostridium botulinum* seen in farmed fish , shellfish and fishery products' is a record of bonafide research carried out by me and it has not previously formed the basis for the award of any other degree ,diploma, associateship, fellowship or other similar titles or recognition.

Cochin-14

March 1998

K. V. LALITHA

CERTIFICATE

This is to certify that this thesis entitled "Ecology and pathogenicity of the anaerobic pathogen *Clostridium botulinum* seen in farmed fish, shellfish and fishery products" is a bonafide record of the research work done by Smt.K.V.Lalitha under my supervision and guidance at Central Institute of Fisheries Technology during the tenure of her Ph.D. (Mariculture) programme (1994-1997). I further certify that this thesis has not previously formed the basis for the award of any degree, diploma or other similar titles or recognition.

Cochin-29
March, 1998

Dr.K.Gopakumar
Supervisor and Chairman,
Advisory Committee

ADVISORY COMMITTEE

Dr. K. Gopakumar Deputy Director General (Fisheries) Indian Council of Agricultural Research New Delhi.	Chairman & Major Advisor
Dr. V.Kunjukrishna Pillai Sr. Scientist, Fishery Environment and Management Division CMFRI, Cochin- 682014.	Co- Chairman
Dr. M.Vijayakumaran Sr. Scientist Madras Research Centre Of CMFRI Tamil Nadu.	Member
Dr. K.S.Schariah Sr. Scientist Fisheries Resources Assessment Division, CMFRI, Cochin- 682014	Member
Dr.V.Sreeramachandra Murthy Principal Scientist & Head , Demersal Fisheries Division CMFRI, Cochin- 682014	Member

ACKNOWLEDGEMENTS

It gives me genuine pleasure to express my deep sense of gratitude to Dr. K.Gopakumar the then director of CIFT Cochin and presently the Deputy Director General (fisheries), ICAR New Delhi, under whose direct guidance this investigation has been carried out. I am grateful to him for his valuable suggestions, constant encouragement and also for providing all facilities at CIFT ,Cochin during the entire course of this investigation.

I am extremely grateful to Dr. M.Devaraj, Director CMFRI for providing all facilities and for his kind permission to carry out the work at the Central Institute of Fisheries Technology Cochin.

I express my sincere thanks to other members of the advisory committee Dr. V.Kunjukrishna Pillai, Sr Scientist; Dr. M.Vijayakumaran, Sr Scientist; Dr. Scariah, Sr Scientist and Dr. V Sreeramachandra Murthy, Principal Scientist for their valuable advice and help.

I am grateful to Dr. P.K. Surendran, Head, Microbiology Fermentation and Biotechnology section, CIFT ,Cochin for providing all facilities and for his valuable advice and help during the course of my investigation. Sincere help and suggestions rendered by Dr.Nirmala Thampuran, Sr.Scientist; Shri.V.N. Nambiar, Sr.Scientist Microbiology Fermentation and Biotechnology section and Dr. T.K. Srinivasa Gopal Sr.Scientist, Fish processing division CIFT, are gratefully acknowledged.

I am thankful to Dr.S.A.H. Abidi, Director and Vice-Chancellor, Central Institute of Fisheries Education, Deemed University, Mumbai for giving me the opportunity to work for my Ph.D in Mariculture at CMFRI, Cochin.

Sincere thanks are due to Dr. Suseelan, Sr. Scientist and officer-in-charge, PGPM, CMFRI Cochin for his valuable suggestions and help.

Technical assistance rendered by M/s. N.M.Vasu, P.Raman Nampoodiri, J.Samarajan, B.Ganesan, A. Indira Devi, K.S. Mythri, K.K. Radhakrishnan, C.Ammini and Sreedharan Technical staff, Microbiology fermentation and Biotechnology Section, CIFT, Cochin and Omanakuttan, Technical staff, Fish processing Division, CIFT is highly appreciated.

K.V. LALITHA.

CONTENTS

1. INTRODUCTION

Aquaculture has become one of the expanding food production method in the world. Its growth rate in the last decade has been phenomenal recording 9.4% per year from 1986 to 1995 (FAO 1977). Global aquaculture production totalled 27.8 mmt and aquaculture production of fish and shellfish reached 20.9 mmt in 1995.

India is a major fish producing country in the world. Aquaculture production of fish and shellfish in India increased from 686260 metric tonnes to 1608938 metric tonnes over the period 1986 to 1995. In aquaculture, India holds the second position in the world.

Aquaculture contributes substantially to world food used for direct human consumption. Percapita food fish supply from aquaculture has increased by 163% since 1984, from 1.4kg to 3.68kg in 1995. With the marine fish production reaching a plateau, any further demand for fish has to be met from aquaculture only. In order to reduce the gap between supply and demand of food fish development of ecofriendly and sustainable aquaculture is very important.

Fish and crustaceans are generally safe food for human. However, the risk of contamination of fishery products by microorganisms, chemicals etc. is greater in farming systems compared to open seas. Food safety hazards due to microorganisms are significant from human health consideration. Bacteriological hazards include pathogenic bacteria associated with food-borne disease. There are two broad groups of pathogenic bacteria of public health significance that will contaminate products from aquaculture - those that are indigenous to the aquatic environment from which fish/shellfish are

harvested and those introduced into fish/shellfish as a result of environmental contamination resulting from disposal of sewage and land run-off. In addition to this, pathogens are also introduced into fish and fishery products during post harvest handling and processing. One major pathogen associated with food poisoning is *Clostridium botulinum*.

Clostridium botulinum is an ubiquitous spore forming anaerobic organism that produces powerful neutrotoxin causing life threatening food-borne illness called botulism in man and bankruptcy disease or fish botulism in fish. This organism can be grouped into seven types A to G based on the antigenic nature of the neutrotoxins that are produced.

The species *C. botulinum* involves at least four biologically different groups: Group I (all type A, some type B and F strains that are strongly proteolytic), Group II (all type E and some type B and F strains that are non-proteolytic but strongly Saccharolytic), Group III (all type C and D strains) and Group IV (only one strain of type G that is proteolytic but non-saccharolytic). Botulism has been known to follow wound infections and recently infant botulism has resulted from establishment of *C. botulinum* in the neonate intestine.

Clostridium botulinum is naturally found in aquatic environments and is often encountered in fish, particularly in farmed fish and shrimp. These indigenous pathogenic bacteria when present in fresh cultured products, are usually found at fairly low levels and where these products are adequately processed, food safety hazards are insignificant. However, if the fish are not properly handled and processed, the organism will grow, multiply and produce toxins with risk to human health.

One of the research needs for production of safe farmed fish is to fill the gap in our knowledge in the occurrence, nature and survival of *C. botulinum* in farmed fish. This involves the study of their growth and survival in aquaculture systems. The ecology of *C. botulinum* is not fully documented in India. The present investigation is carried out to identify the bacteria (*Clostridium botulinum*), investigate the type, its survival under various processing conditions and suggest practical control measures to produce a safe fish fit for human consumption. It is also aimed at to augment a good aquaculture practice to produce farmed fish free from the toxin of *C. botulinum.*

2. REVIEW OF LITERATURE

2.1 Anaerobic bacteria in fish farms

The obligately anaerobic bacteria are large and diverse group of microorganisms, which occur and often predominate in a variety of natural habitats. Whilst those associated with human beings are relatively well documented, increasing attention is now being given to anaerobes in other habitats including the aquatic environments, foods and animals in health and disease. Many aerobic and anaerobic bacteria seen in fish could be derived from their environment (Horsley 1973) and diet (Trust 1971). They often occur predominantly in fish culture ponds contributing to mineralisation (Sugita *et al.* 1989). In fish farming, organic matters either introduced into or produced in the culture water are partially mineralized by heterotrophic bacteria. The heterotrophic bacteria of fish farms have been extensively studied (Acuff *et al.* 1984; Sugita *et al.* 1985; Fonseka 1990; Llobrerra *et al.* 1990; Nedoluha and Westhoff 1993; Nayyar Ahamed *et al.* 1994; Surendran *et al.* 1994; Sharmila *et al.* 1996). Anaerobic bacteria are the predominant organisms in many habitats such as soil, sediments, fish farms and gastrointestinal tracts of many animals (Sakata *et al.* 1980; Sugita *et al.* 1985, 1988, 1989; Wibowo *et al.* 1991; Inglis *et al.* 1993; Nedoluha and Westhoff 1993) playing a vital role in the digestion of waste and in environmental pollution (Barns and Mead 1986). Anaerobic bacteria have also been implicated as fish pathogens (Austin and Austin 1993). In farm environments, adverse environmental conditions and farming practices may favour the multiplication of pathogens and contamination of the farmed species. One of the major concerns in aquaculture are environmental contaminants (Pillay 1992). Many anaerobes including Clostridium have been isolated from fish.

Clostridia are found to be commonly present in soil, sewage, marine sediments (Davies 1969; Smith 1975; Sneath 1986), on whole fish (Shewan 1967, 1977; Barns 1986; Lalitha and Iyer 1986; Lalitha *et al.* 1990) and in commercial pelletted diets used in farms (Trust 1971; Sugita *et al.* 1989). Among clostridia, *Clostridium botulinum* remains the single accredited and serious pathogen of fish (Roberts 1989; Sindermann 1990; Inglis *et al.* 1993; Fryer and Rohovec 1993). *C. botulinum* grows well in both fish and humans and are able to cause disease in both. A few nanogram (0.5-5 ng) of toxin is sufficient to cause disease in humans (Notermans *et al.* 1994, 1995).

2.2 *Clostridium botulinum* in soil and sediments

The prevalence of *C. botulinum* in soil and sediments has been the subject of a number of reviews (Dolman 1957, 1964; Hobbs 1976, 1981; Sakaguchi 1979; Huss 1981; Barnes and Mead 1986; Smith 1990; Dodds 1993a). In some areas of the world, soil or sand is so heavily contaminated that almost every sample taken will contain it (Roberts and Lund 1991).

Soil surveys revealed the predominance of type A *C. botulinum* in the Western United States, Brazil and Argentina and proteolytic type B in the Eastern United States, types C, D, E, F and G also occur (Sakaguchi 1979; Smith 1990; McClure *et al.* 1994). In South America, *C. botulinum* types A, C, F and G were detected in soil (Halbinger 1973; Sakaguchi 1979; Hayashi *et al.* 1990). Extensive surveys of mud collected from the Pacific coast, U.S. Atlantic coast, East coast of the U.S., Canada, Gulf of Mexico and more southern Atlantic oceans detected types A through F (Pressnell *et al.* 1967; Ward *et al.* 1967 a, b, c; Hobbs 1981). Eklund *et al.* (1982, 1984) recovered type E spores at

high levels from farm sediments following an outbreak of botulism in juvenile coho salmon in the United States. Bott *et al.* (1968), Sugiyama *et al.* (1970) and Halbinger (1973) estimated the numbers of *C. botulinum* in freshwater sediments from Green Bay and rivers. Eklund and Poysky (1967) and Nickerson *et al.* (1967) have reported the level of contamination of sediments from the U.S. East coast and the Pacific coast.

The prevalence of *C. botulinum* types A, B, C, D and E were reported in the soil surveys conducted in the U.K., Scotland and Europe (Smith and Young 1980; Smith 1987, 1990; Lund 1986; Ortiz and Smith 1994). Incidence of *C. botulinum* in sediment samples from lakes and water ways in London, Norfolk Broads, Scandinavia, Denmark, U.K., Faroe Islands, Iceland, coastal waters of the Skagerrak, the Kattegat, the Sound and the Baltic has been extensively studied (Huss 1980; Hobbs 1981; Lund 1986; Smith 1990). Huss (1981) estimated the numbers of *C. botulinum* in sediments from Scandinavian waters and in the freshwater sediments from Denmark. Work carried out on the freshwater trout farms in Germany, Scandinavia, Scotland, Denmark and the U.K. showed occurrence of psychrotrophic strains of types B, E and F in some cases at high levels (Bach *et al.* 1971; Huss *et al.* 1974 Burns and Williams 1975; Cann *et al.* 1975; Cann and Taylor 1982, 1984). Smith (1987, 1990) reported incidence of *C. botulinum* in freshwater sediments in the U.K. In the former U.S.S.R., except type D, *C. botulinum* types A-F were prevalent in the soil and sediment samples (Hobbs 1976; Sakaguchi 1979; Huss 1981).

In Asia, soil surveys conducted in Japan (Sakaguchi 1979; Otofuji *et al.* 1987; Smith 1990), Indonesia (Hayashi *et al.* 1981), the Gulf of Thailand (Tanasugarn 1979), Sri Lanka (Wijiwanta 1962) and India (Pasricha and Panja 1940) demonstrated

the occurrence of *C. botulinum*. The incidence of *C. botulinum* was reported in freshwater sediments from Japan (Hobbs 1976; Azuma and Itoh 1987; Venkateswaran *et al.* 1989 b), Indonesia (Haq and Suhadi 1981), Bangladesh (Huss 1980) and brackishwater sediments from Bangladesh (Huss 1980) and India (Lalitha and Surendran 1993) and marine sediments from Japan (Huss 1981, Venkateswaran *et al.* 1989 a), Iran (Huss 1981), Indonesia (Mortojudo *et al.* 1973; Haq and Suhadi 1981; Suhadi *et al.* 1981) and the Gulf of Thailand (Tanasugarn 1979).

In South Africa, survey of soil (Kamiya *et al.* 1990; Smith 1990) and sediments (Smith 1987, 1990) revealed the prevalence of *C. botulinum* type D. In Egypt, survey of mud and water from fish ponds and lakes did not yield any with *C. botulinum* (Weber *et al.* 1993). In Australia, *C. botulinum* was isolated from soil and survey of mud samples did not yield any *C. botulinum* type E (Eyles and Warth 1981; Huss 1981).

2.3 *Clostridium botulinum* in fish and shellfish

2.3.1 Wild fish and shellfish

The aquatic environment is highly contaminated with *C. botulinum*. Therefore, it is to be expected that fish will often harbour them. The distribution of *C. botulinum* in fish has been extensively studied and reviewed by Dolman (1957) and others (Hobbs 1976, 1981; Sakaguchi 1979; Huss 1981; Smith 1990; Dodds 1993).

Incidence of *C. botulinum* in fish was reported from the Great Lakes (Bott *et al.* 1966), Pacific coast (Craig and Pilcher 1967; Eklund and Poysky 1965, 1967; Craig *et al.* 1968), U.S. Atlantic coast, Gulf of Mexico and more southern Atlantic oceans (Carroll *et al.* 1966; Ward and Carroll 1965; Ward *et al.* 1967 a, b, c) and eastern coast of the United States and Canada (Nickerson *et al.* 1967; Williams-Walls 1968) and inland lakes

(Chapman and Naylor 1966). Llobrera (1983) found none of the 175 commercial fish and shellfish samples harvested from the Gulf of Mexico and Pacific coast to be contaminated with *C. botulinum*. In contrast, Baker *et al.* (1990) found contamination in six out of the 28 commercial fish and shellfish samples from the Pacific coast.

With the exception of Scandinavia, the level of contamination of fish in Europe appears to be much lower than that in the United States (Dodds 1993 a). A survey of fish samples from North sea, England and Wales did not yield any with *C. botulinum* (Cann *et al.* 1967; Huss 1981). Incidence of *C. botulinum* in fish was reported from Baltic sea, Scandinavia, Skagerrak and North sea (Cann *et al.* 1965 a, 1966, 1968, 1975; Huss and Pedersen 1979; Zaleski 1981). *Clostridium botulinum* was detected in fish from Germany (Baumgart 1972). *Clostridium botulinum* was not found in wild freshwater fish from Europe (Huss 1981).

In the former USSR, *C. botulinum* type E was reported to be the predominant type in fish (Bulatova *et al.* 1973; Hobbs 1981).

In Asia, incidence of *C. botulinum* was reported in fish from Japan (Kanzawa *et al.* 1970; Yamamoto *et al.* 1970; Azuma and Itoh 1987), Indonesia (Mortojudo *et al.* 1973; Haq and Suhadi 1981), the Gulf of Thailand (Tanasugarn 1979) and India (Lalitha and Iyer 1990).

In South Africa, *C. botulinum* was not found in fish from Egypt (Weber *et al.* 1993). In Australia, a survey of fish did not yield any with *C. botulinum* (Eyles and Warth 1981)

2.3.2 Farmed fish

There have been few surveys for *C. botulinum* in farmed fish. An outbreak of

human botulism in Germany caused by smoked, farm-raised trout has drawn the attention to the botulinogenic potential of farm-raised trout (Bach *et al.* 1971) and resulted in the studies to assess the incidence of *C. botulinum* in trout farms in Germany (Wenzel *et al.* 1971), Denmark (Huss *et al.* 1974), Scotland (Burns and Williams 1975) Great Britain (Cann *et al.* 1975) and Finland (Ala Huikku *et al.* 1977). Botulism or Bankrupt disease as a major cause of fish mortality in pond-reared trout was first reported in Denmark by Huss and Eskildson (1974) and later in Britain in 1980 (Cann and Taylor 1982, 1984) and in the United States (Eklund *et al.* 1982, 1984).

Huss *et al.* (1974) estimated the numbers of *C. botulinum* in farmed fish. Cann and Taylor (1982, 1984) estimated the toxin levels in the sick fish. Baker *et al.* (1990) in a survey on *C. botulinum* in farm-raised catfish and King Salmon from Nimbus hatchery estimated the level of contamination in King Salmon. *C. botulinum* was detected in none of the samples in a survey of aquacultured hybrid striped Bass from Maryland (Nedoluha and Westhoff 1993).

Sensitivity of fish to *C. botulinum* toxins was reported earlier (Crisley 1960; Skulberg and Grande 1967; Hiroki 1970; Haagsma 1975). According to Skulberg and Grande (1967) type E proved to be the most poisonous. Cann and Taylor (1982) and Eklund *et al.* (1984) while investigating the conditions contributing to fish botulism outbreaks, reported sensitivity of fish to type E toxin.

2.4 Clostridium *botulinum* in fishery products

Contamination of fish with *C. botulinum* occurs while a product is growing or being harvested and is, therefore, more likely when a product originates in an environment where the incidence of spores is high. However, contamination can also

occur during and after processing unless foods are adequately preserved.

Earlier survey of fishery products in the United States revealed contamination by *C. botulinum* in products such as smoked fish (Pace *et al.* 1967, Fantasia and Duran 1969; Hayes *et al.* 1970), commercially produced haddock fillets (Goldblith and Nickerson 1965), commercial vacuum packed frozen flounder (Insalata *et al.* 1967), frozen packaged fish (Thatcher *et al.* 1967), canned salmon (Houghtby and Kaysner 1969) and retail fish (Post *et al.* 1985). In California, *C. botulinum* type E was not detected in any of the 35 samples of commercial seafood cocktails (Lerke 1973). Abeyta (1983) did not detect *C. botulinum* in the 287 samples of shellfish and finfish evaluated. In a later survey, Lilly and Kautter (1990) found none of the 1074 samples of commercial vacuum-packed fresh fish in the United States to be contaminated with *C. botulinum*. Baker *et al.* (1990) found an overall contamination level of 22% and 67% in two surveys of fresh fish and other seafoods from California super market. In a survey of both hot and cold smoked ready to eat fish at the retail level in Toronto region, *C. botulinum* was not detected in any of the 100 samples analysed (Dodds *et al.* 1992).

In Europe, with the exception of Scandinavia, the level of contamination of prepared fish appears to be much lower than that in the United States (Dodds 1993 b). Survey of fishery products in Europe demonstrated *C. botulinum* in vacuum-packed fish in England (Cann *et al.* 1965 b, 1966), in smoked herring and trout in Sweden (Johannsen 1965), in smoked salmon in Denmark (Nielsen and Pedersen 1967), in smoked Eel in the Baltic area (Abrahamsson 1967), in smoked trout fillet in Germany (Wenzel *et al.* 1971; Back *et al.* 1971) and in fresh and vacuum-packed trout at the retail shops in the U.K. (Jarvis and Patel 1980). *C. botulinum* was not detected in vacuum-packed fish in

England (Hobbs *et al.* 1965). Gibbs *et al.* (1994) found none of the 500 refrigerated packaged smoked and raw fish currently in sale in the U.K. to be contaminated with *C. botulinum.*

In Asia, *C. botulinum* was detected in salted and smoked carp in Iran (Rouhbakhsh-khaleghdoust 1975), in commercially prepared and home made izushi in Japan (Kanzawa *et al.* 1970), in trout products originated from Japan and sold in the U.K. (Jarvis and Patel 1980) and in fish sold at the retail shops in Osaka (Haq and Sakaguchi 1980). *Clostridium botulinum* was demonstrated in fish and sea foods from commercial fishermen, Pindang and boiled fish from various markets in Indonesia (Suhadi *et al.* 1981) and in fish and cured fish sold at the retail markets in Cochin, India (Lalitha and Surendran 1992, 1993). However, Rouhbakhsh - Khaleghdoust (1975) failed to detect *C. botulinum* in any of the 17 samples of salted and smoked fish in Iran.

2.5 Fishery products and Botulism outbreaks

Botulism is widely distributed, but its real prevalence in the world is unknown as nearly all our information comes from the more highly developed countries. Numerous outbreaks (nearly 3390 outbreaks) of human botulism have been recorded in the world during the last four decades with food identified. Fish and fishery products have been implicated in 14% of all outbreaks. 91 percent of the botulism outbreaks were associated with home-preserved products whereas commercially processed products were involved in only 9 percent of the outbreaks (Hauschild 1993). In the United States and China, vegetables are the main source of botulism. In Poland and continental Europe (apart from Spain and Italy), meat is the main source. In Alaska, Norway, Sweden, Japan, Iran and

the former Soviet Union, the majority of the outbreaks were associated with fish. Sakaguchi (1979); Licciardello (1983); Hauschild (1993) and Todd (1997) have extensively reviewed the recorded food-borne botulism cases. Almost all type E outbreaks were due to fish, fish eggs or sea mammals all over the world (Sakaguchi 1979). Fresh fish have never been implicated in human botulism (Huss 1994). In a review of the food safety hazards, Reilly and Käferstein (1997) have indicated that although botulism associated with aquacultured fish is very rare, it should not be overlooked in carrying out a hazard analysis.

In the United States, of the 39 outbreaks reported from fish, 25 occurred in Alaska alone. In fish-borne botulism outbreaks, canned tuna fish from Detroit (Johnston *et al.* 1963), commercial smoked white fish chubs from Great Lakes (Sakaguchi 1979), commercially produced salt-cured air dried uneviscerated white fish (Kapchunka) in the United States (McClure *et al.* 1994) and fish from Hawaii and Puerto Rico (Hauschild 1993) were implicated. In Canada, fish was implicated in 20 percent of the 75 botulism outbreaks. Raw, parboiled or dried fish, raw or undercooked flesh of seal, whale or walrus, fermented salmon eggs and smoked fish were identified as the responsible foods in the above outbreaks (Hauschild 1993).

In South America, outbreaks of fish borne botulism have been reported only from Argentina, Mexico and Brazil (Hauschild 1993). Pickled trout and carp from Argentina, canned tuna from Mexico and home-preserved fish from Brazil were implicated in the outbreaks. Reports of botulism from other Latin American countries have been sporadic.

In Europe fish-borne botulism outbreaks have been reported from Norway, Poland, Germany, Belgium, Czechoslovakia, Italy, Portugal and Spain. Most of the

implicated fishery products were commercially canned in Poland, home-preserved fish in Czechoslovakia and Germany, smoked trout fillet in Germany, imported Alaskan salmon in cans in Belgium, home-preserved fish in Italy (Hauschild 1993), rake fish (raw fermented trout) in Norway, smoked fish in Denmark, fish products in Sweden, pickled fish and canned salmon in Britain (Hauschild 1993). In Austria, Greece, Netherlands, Finland, Northwales, Ireland and Iceland, outbreaks of fish-borne botulism have not been recorded till 1989.

In the former U.S.S.R., fish-borne botulism outbreaks have been recorded in the period 1958-64 and afterwards epidemiological work on botulism has been discontinued. Home preserved fish were implicated in two third of the total 83 outbreaks (Hauschild 1993).

In Asia, fish-borne botulism has been reported only from a few countries. Fishery products implicated in the outbreaks included ribbetz or kapchunka (brined air-dried freshwater fish) processed in New York and brought to Israel (Stater *et al.* 1989; Telzak *et al.* 1990), ashbal (home-preserved fish eggs) in Iran, 'izushi' and Kirikomi in Japan (Owens and Mendoza 1985; Hauschild 1993). Fish-borne botulism has not been reported from India, Bangladesh, Thailand, Indonesia, Taiwan and China.

In Africa, the first recorded fish-borne botulism occurred in 1982 (Hauschild 1993). In Egypt, faseikh - a traditional salted fish, was implicated in botulism outbreak (Weber *et al.* 1993).

In Australia, only one fish-borne botulism outbreak has so far been reported in the period 1942 to 1983 associated with canned tuna. An outbreak of botulism was also

reported in New Zealand. The incriminated food was a batch of home bottled fermented mussels mixed with watercress, a traditional Maoridish (West 1989; Hauschild 1993).

Clostridium botulinum types A, B and E are the common types involved in human botulism and type F is involved to a lesser extent. Hauschild (1993) reported that because of very low toxicity to primates, the presence of toxins C and D is probably not a significant factor in pathogenesis. However, types C and D have both been implicated in earlier outbreaks (Roberts and Gibson 1979; Hauschild 1993); but either type as the cause of human botulism is in some doubt (Hauschild 1993). Sonnabend and Sonnabend (1981) found *C. botulinum* type D and toxin in patients who died unexpectedly with symptoms like respiratory difficulty and severe dysphagia. Fastidiousness of type C in experimental monkeys (Dolman *et al.* 1961) and an outbreak of type C botulism in captive monkeys have also been reported (Smith 1990). Recently, a case of infant botulism by type C was reported in Japan (Oguma *et al.* 1990).

2.6 Stability of *Clostridium botulinum* toxin

The stability of *C. botulinum* toxins at various temperatures has been extensively studied (Scott 1950; Scott and Stewart 1953; Prevot and Brygoo 1953; Cartwright and Lauffer 1958; Abrahamsson *et al.* 1965; Licciardello *et al.* 1967 a, b; Losikoff 1978; Woolford *et al.* 1978; Bradshaw *et al.* 1979; Roberts and Gibson 1979; Lewis 1981). Cardella *et al.* (1960) have reported high toxicity of botulinum type D toxin after fractionation by series of alcohol precipitations and a potentiating activity of gelatin phosphate dilutent on the type D toxin. Woodburn *et al.* (1979) have shown that the substrate in which toxin is heated has a marked effect on the inactivation rate. Yao *et al.*

(1973); Woolford *et al.* (1978) and Hubalek and Halouzka (1988) reported that the toxin of *C. botulinum* is unaffected by freezing. Stability of botulinum toxin to frozen storage has been reported by several investigators (Georgala and Hurst, 1963; Genigeorgis and Riemann 1979; ICMSF 1980; Huss 1981) Botulinum toxin once formed is stable at chill temperature and at acidic pH (Sakaguchi 1979). Standing at room temperature at pH 6.2 or 6.8 results in a gradual loss of toxicity (Lewis 1981). Roberts and Gibson (1979) have reported lower stability of botulinal toxins in simple buffers than in buffered systems containing gelatin. Huss and Peterson (1980) have shown the stability of type E toxin at room temperature in sterile culture filtrate or spoiling fish and in low acid fish products and toxin is unaffected by sterile saturated salt solutions and in salted fish. Wheaton and Lawson (1985) reported that the toxin is easily destroyed by heat (2 min at 70°C). Hubalek and Halouzka (1988) studied thermal sensitivity of *C. botulinum* type C toxin.

2.7 Control of *Clostridium botulinum* in fish and fishery products

Control of *C. botulinum* in fish and fishery products is achieved by inhibition of growth and toxin production of *C. botulinum*. The growth of *C. botulinum* in foods depends on a number of limiting factors such as temperature, pH, water activity, redox potential, gaseous atmosphere, added preservatives and competing microflora and the inhibitory effects of those factors alone on growth and toxin production of *C. botulinum* are well documented (Sperber 1982; Eklund 1993; Gaze 1992; McClure *et al.* 1994). Lynt *et al.* (1982) reviewed the differences and similarities among proteolytic and non-proteolytic strains of *C.* botulinum types A, B, E and F. Psychrotrophic *C. botulinum* can grow at temperatures at which fish and fish products are normally held and this has raised

concerns over the possibility of unchecked growth of these organisms under some methods of pre-processing and packaging, increasingly common in modern food retailing (Liston 1990).

2.7.1 Temperature

Several investigations have been undertaken in the past to define the range of temperatures which permit initiation of growth and toxigenesis in culture media and foods by the various serotypes of *C. botulinum* (Ohye and Scott 1953, 1957; Schmidt *et al.* 1961; Eklund *et al.* 1967 a, b; Roberts and Hobbs 1968; Segner *et al.* 1971; Solomon *et al.* 1977, 1982; Smelt and Has 1978; Sugiyama 1980; Sperber 1982; Jensen *et al.* 1987; Eklund 1993; Graham and Lund 1993; Lund 1993). Strasdine and Kelly (1967), Boyd and South cott (1968), Lerke and Farber (1971), Solomon *et al.* (1977, 1982), Patel *et al.* (1978), Huss (1981), Goldmintz *et al.* (1983) and Fletcher *et al.* (1988) emphasised the way in which growh temperatures can be influenced by the substrate. The spores of *C. botulinum* appear to be completely resistant to freezing (Cardella *et al.* 1960; Georgala and Hurst 1963). Studies have been conducted in the kinetics of toxin production by changing the temperature, pH or some other conditions. (Bonventre and Kempe 1960; Siegel and Metzger 1979, 1980; Siegel 1981).

2.7.2 pH

Acidification (low pH) is used widely to control growth and toxin production of *C. botulinum* in food products (Sperber 1982; Eklund 1982, 1993). Segner *et al.* (1966) and Lerke (1973) studied the pH range for growth of *C. botulinum* in a variety of culture media and crab cocktail. Odlaug and Pflug (1978), Raatjes and Smelt (1979), Smelt *et al.*

(1982), Lund *et al.* (1987), Young Perkins and Merson (1987) and Dodds (1989) demonstrated growth and toxin production of *C. botulinum* at pH values below 4.6 in laboratory media or under laboratory conditions where there is high concentration of proteins present and various acidulants used. Growth of a mould (Odlaug and Pflug 1979) or of *Bacillus licheniformis* (Montville 1982) in an acidic food may elevate the pH to a value higher than 4.6 and allow growth of *C. botulinum*. Several investigators have shown that the inoculum size, type of strains used, incubation temperature and medium utilized affects the minimum pH at which outgrowth of *C. botulinum* in a food product will occur (Lerke 1973; Ito and Chen 1978; Smelt *et al.* 1982; Wong *et al.* 1988). Roberts and Gibson (1979) reported that the minimum pH for growth and toxin production of type C was similar to or slightly higher than that for types A, B and E.

2.7.3 Water activity (a_w)

Reducing a_w is a well known way to improve the microbial stability of foods (ICMSF 1980; Richard Molard and Lesage 1986). The primary influence of a_w on growth and toxin production of *C. botulinum* was investigated by Segner *et al.* (1966, 1971 a, b), Baird and Parker (1967), Ohye and Christian (1967), Emodi and Lechowich (1969) and Roberts and Ingram (1973). Roberts and Gibson (1979) reported type C as more sensitive to sodium chloride than types A, B or E. Huss and Petersen (1980) showed stability of *C. botulinum* type E toxin for weeks at room temperature in saturated salt (NaCl) solutions and in salted fish. Lund and Wyatt (1984) studied the interaction of redox potential with sodium chloride concentration on the probability of growth of *C. botulinum* type E. Sperber (1982), Eklund (1982, 1993) and Mc Clure *et al.* (1994)

reviewed the combination of water activity and salt concentration minimum values for growth and toxin production of *C. botulinum* and the factors important when considering the minimum a_w for growth.

2.7.4 Gaseous atmosphere

Modification in gaseous composition during storage has been promoted as a means of extending the storage life of fresh fishery products (Finne 1982; Cann *et al.* 1983, 1984; Genigeorgis 1985; Hotchkiss 1988; Gopal *et al.* 1990, 1996; Reddy *et al.* 1992). Several investigations have demonstrated that vacuum packaging is not a requirement for *C. botulinum* growth in fish (Cann *et al.* 1980; Eyles and Warth 1981; Huss 1981; Eklund 1982; Penny *et al.* 1994). Schofield (1992) reviewed the significance of *C. botulinum* in chilled vacuum-packed foods and the potential hazard.

Studies attempting to assess the risk of *C. botulinum* toxigenesis in fish stored at low and abused temperatures under a variety of Modified Atmosphere have been reported (Cann *et al.* 1965, 1980; Huss 1981; Stier *et al.* 1981; Eklund 1982; Post *et al.* 1985; Lindroth and Genigeorgis 1986; Ikawa and Genigorgis 1987; Garcia and Genigeorgis 1987; Garcia *et al.* 1987) and in a model broth system (Jensen *et al.* 1987). Farber (1991) reviewed the microbiological aspects of Modified Atmosphere Packaging (MAP) Technology. Several investigations on pre-treatment of fishery products before vacuum or MAP have been reported to improve the shelf life (Reddy *et al.* 1992;). MAP in combination with low temperature storage may not provide the safety required for extended storage of fresh fish products with respect to out growth and toxin production by non-proteolytic strains of *C. botulinum* (Reddy *et al.* 1992; Gibbs *et al.* 1994). Reddy *et al.* (1996), while investigating the potential for toxin development by *C. botulinum*

type E in pond raised tilapia fillets concluded that Modified Atmosphere Packed and vacuum packed fish held to severe temperature abuse condition may pose a public health hazard.

2.7.5 Combination effects

A number of studies have examined the effects of combinations of factors such as pH, a_w , temperature against growth and/or toxin production of *C. botulinum* (Segner *et al.* 1966; Baird-Parker and Freame 1967). Less salt is needed under acid conditions to inhibit growth of *C. botulinum* (Roberts and Ingram 1973). Hauschild *et al.* (1982) combined different brine and nitrite concentrations and temperature to monitor the effects of combinations on *C. botulinum* present in liver sausage. Lund *et al.* (1985) and Graham and Lund (1987) investigated the combined effect of temperature and pH on the growth of *C. botulinum*. Fermented aquatic products depend primarily on low pH and high salt content to prevent growth of *C. botulinum* (Wheaton and Lawson 1985). Lund *et al.* (1990) monitored the combined effect of pH, temperature and preservative on the growth of *C. botulinum*. Graham *et al.* (1996) studied the inhibitory effect of combinations of heat treatment, pH and sodium chloride on growth from spores of non-proteolytic *C. botulinum* at refrigeration temperature. The effect of additional barriers such as starter cultures of Lactic acid bacteria, bacteriocins or essential oils on the inhibition of growth and toxin production were also evaluated (Okereke and Montville 1991; Crandall and Montville 1993; Chaibi *et al.* 1997).

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Media

The bacteriological media used in the study included dehydrated media and media compounded in the laboratory.

3.1.1.1 Dehydrated media

Dehydrated media supplied by Oxoid, England were used. The media utilized were fluid thioglycollate medium and Baird-Parker Agar.

3.1.1.2 Media compounded in the laboratory

Cooked meat medium (CMM)

Ingredients

A	Beef heart	500 g
	Distilled water	1000 ml
	Sodium hydroxide (1N)	25 ml

B.	Tryptone	10.0g
	Proteo se peptone	20.0g
	Yeast extract	5.0g
	Sodium chloride	5.0g
	D-Glucose	4.0g
	Sodium thioglycollate	1.0g
	Soluble starch	2.0g

Cooked meat medium (modified) was prepared according to the method outlined in FDA bacteriological analytical manual (1995). Fresh beef heart was used instead of dehydrated beef infusion. pH was adjusted to 7.4. Autoclaved at 121°C for 15 min.

Trypticase-peptone glucose yeast extract (TPGYB) broth

Ingredients

Tryptone	50.0g
Bacto-peptone	5.0g
Yeast extract	20.0g
Dextrose	4.0g
Sodium thioglycollate	1.0g
Distilled water	1000ml

The medium was prepared according to FDA Bacteriological Analytical Manual (1995). Immediately before use, boiled 10-15 min to remove oxygen and cooled quickly.

For the growth of *C. botulinum* type E, aseptically added 1ml of trypsin solution per15ml Broth

Prepared trypsin solution by dissolving 15g trypsin (Difco 1:250) in 100ml distilled water. Sterilized by filtering through 0.45 μm Millipore filter and refrigerated.

Tryptone soytone glucose yeast extract (TSGYB) broth

Ingredients

Tryptone	50.0g
Soytone	5.0g
Yeast extract	20.0g
Dextrose	5.0g
Distilled water	1000 ml

Dispensed 15ml portions into 20x150mm culture tubes Autoclaved 15min. at 121°C. Final pH 7.0±0.1.

Aseptically added 0.15ml glucose (50%) solution per 15ml broth after sterilization.

50% glucose solution was prepared and sterilized by filtering through 0.2μm Millipore filter.

Tryptone soytone glucose yeast extract agar (TSGYA)

Ingredients

Tryptone	50.0g
Soytone	5.0g
Yeast extract	20.0g
Dextrose	50.0g
Sodium bicarbonate	1.0g
Agar	20.0g
Distilled water	900 ml
Egg yolk emulsion (50%)	100 ml
Hemin	0.005g
L-cysteine hydrochloride	0.4g
Vitamin K	0.001 g

All ingredients except glucose, hemin, vitamin K, L-Cysteine hydrochloride and egg yolk emulsion were dissolved and the pH was adjusted to 7.2±0.1 and autoclaved for 15min at 121°C according to the method outlined by Lalitha and Surendran (1993).

50% sterile glucose solution was prepared by filtering through 0.2µm millipore filter and aseptically added to the tempered medium.

Dissolved the hemin and the L-cysteine hydrochloride in 5ml of 1N NaOH, filter sterilized and added to the tempered medium.

Added vitamin K from a filter sterilized stock alcoholic solution containing 1g of 3 phytylmenadione (Vitamin K) plus 99ml of absolute alcohol.

50% Egg yolk emulsion was prepared according to the method followed by FDA Bacteriological Analytical Manual (1995).

Peptone Yeast extract (PY) basal medium

Ingredients

Tryptone	0.5g
Peptone	0.5g
Yeast extract	1.0g
Vitamin K1 (1% solution)	0.1ml
Hemin (1% solution)	0.1ml
L-cysteine hydrochloride	0.05g
Salt solution*	4ml
Distilled water	100ml

*Salt solution

$CaCl_2$	0.2g
$MgSO_4$	0.2g
K_2HPO_4	1.0g
KH_2PO_4	1.0g
$NaHCO_3$	10.0g
NaCl	2.0g

Mixed $CaCl_2$ and $MgSO_4$ in 300ml distilled water until dissolved. Added 500ml distilled water and swirled the liquid slowly while adding remaining salts. Added 200ml distilled water. From this stock solution added 4 ml to 100ml medium.

The medium was prepared according to the method followed by Dowell *et al.* (1981) and as modified by Sneath (1986).

10% solutions of sugars glucose, sucrose, Lactose, salicin, raffinose, Mannose,

Maltose, galactose, melibiose and fructose were prepared, filter sterilized and added to PY basal medium aseptically to give a final concentration of 1%. For hydrolysis of esculin, esculin 1% and ferric ammonium citrate 0.5% were prepared, filter sterilized and aseptically added to PY basal medium to give a final concentration of 0.1% and 0.05% respectively.

Iron milk medium

Non-homogenized milk	200ml

The medium was prepared according to the method followed by Dowell *et al.* (1981). Placed few iron fillings in the bottom of the tubes. Added 7-8 ml milk to each tube. Autoclaved at 116°C for 20 min.

Trypticase yeast extract glucose (TYG) medium.

Ingredients

Trypticase	3.0%
Yeast extract	2.0%
Glucose	0.4%
L-Cysteine hydrochloride	0.1%

The medium was prepared according to the method followed by Eklund and Poysky (1972) and as modified by Strom *et al.* (1984).

Reinforced Clostridial Medium (RCM Agar)

Ingredients

Peptone	10.0g
Lab lemco powder	8.0g
Yeast extract	2.4g
Cysteine hydrochloride	0.5g
Glucose	5.0.g
Sodium acetate anhydrous	5.0g
Soluble starch	1.0g
Agar	15.0g
Distilled water	1000 ml

The medium was prepared according to the method followed by Barns (1985).

Tryptose sulfite neomycin (TSN) agar

Ingredients

Tryptose	15.0g
Soytone	5.0g
Yeast extract	5.0g
Sodium metabisulphite	1.0g
Ferric Ammonium citrate	1.0g
Agar	20.0g
Distilled water	900 ml

Adjusted pH to 7.6±0.2. Autoclaved 15 min at 121°C. To each litre of sterile medium, added filter sterilised 1% solution of neomycin sulfate (Willis 1979).

Buffered motility nitrate medium

Ingredients

Beef extract	3.0g
Peptone	5.0g
KNO_3	5.0g
Na_2HPO_4	2.5g
Agar	3.0g
Galactose	5.0g
Glycerol	5.0g
Distilled water	1000ml

The medium was prepared according to the method followed by FDA Bacteriological Analytical Manual (1995).

Lactose gelatin medium

Ingredients

Tryptose	15.0g
Yeast extract	10.0g
Lactose	10.0g
Phenol red (1% solution in 95% ethanol	5.0ml
Gelatin	12.0g
Distilleld water	1000 ml

Adjusted pH to 7.5$\pm$0.2. The medium was prepared according to the method outlined in FDA Bacteriological Analytical Manual (1995).

Plate Count Agar (PCA)

Ingredients

Tryptone	5.0g
Yeast extract	2.5g
Dextrose	1.0g
Agar	15.0g
Distilled water	1000 ml

The medium was prepared as per the method followed by FDA bacteriological analytical Manual (1995).

Medium for testing Hydrogen sulphide production

Ingredients

Peptone	1.0g
Soytone	0.3g
Proteosepeptone	1.0g
Digest serum powder	1.35g
Yeast extract	0.5g
Meat extract	0.22g
Liver extract	0.12g
KH_2PO_4	0.25g
NaCl	0.3g
Soluble starch	0.5g
Cysteine - HCl	0.03g
Sodium thioglycollate	0.03g
$FeSO_4$ $7H_20$	0.015g
$Na_2S_2O_35H_20$	0.03g
Agar	0.75g
Distilled water	100 ml

The medium was prepared according to the method followed by Oguma *et al.* (1986).

KF agar

Ingredients

Proteose peptone	1%
Yeast extract	1%
Sodium chloride	0.5%
Sodium glycero phosphate	1%
Maltose	2%
Lactose	0.1%
Sodium azide	0.04%
Sodium carbonate	0.064%
Bromocresol purple (0.1% solution)	1.5ml
Agar	2%
Distilled water	100 ml

adjusted pH to 7.2±0.1 and autoclaved 30 min at 121°C. To each 100ml of the cooled medium, 1ml of 1% 2.3.5 triphenyl tetrazolium chloride (TTC) was added before pouring into the plates.

Mac Conkey (MC) broth

Ingredients

Peptone	20.0g
Lactose	10.0g
Bile salts (No.3)	5.0g
Sodium chloride	5.0g
Bromocresol purple	0.015g
Crystal violet	0.001g
Distilled water	1000 ml

Adjusted pH to 9.4 and autoclaved 20 min at 115°C. Double strength and single strength MC broth were prepared according to the method outlined by Harrigan and Mc Cance (1976).

Gelatin phosphate (GP) Buffer

Ingredients

Gelatin	2.0g
Na_2HPC_4	4.0g
Distilled water	1000 ml

The buffer was prepared according to the method followed by FDA Bacteriological Analytical Manual (1995).

Physiological saline solution

Ingredients

Sodium chloride	8.5g
Distilled water	1000ml

The diluent was prepared as per the method followed by FDA Bacteriological Analytical Manual (1995).

3.1.2 Chemicals

Ingredients such as peptone, proteose peptone, tryptone, beef extract, yeast extract, agar powder, gelatin, Trypsin (1.250) and Esculin for bacteriological media and reagents were supplied by Oxoid (England) and Difco (USA). Gas generating kit, catalyst and anaerobic indicator were supplied by Oxoid (England).

Clostridium botulinum types A-F monovalent antitoxins and polyvalent antitoxin

were obtained from Centre for Disease Control, Atlanta, Georgia.

Chemicals such as neomycin sulphate, fructose, galactose, melibiose, Raffinose, salicin, vitamin K and Hemin were obtained from Sigma Chemicals, U.S.A. Other chemicals used were of BDH (England), Sisco (India), E-merk (India), Himedia (India) brands. Paraffin oil was supplied by Sd Fine Chemicals (India).

3.1.3 Mice

Swiss Webster mice obtained from NIN, Hyderabad and reared in the Institute animal house were used for toxicity test and toxin typing

3.1.4 *Clostridium botulinum* strains

C. botulinum cultures used in the study included (1) strains isolated and identified in course of this work. They were maintained in CMM. (2) Strains of *C. botulinum* type A (NCIB 10640), B (NCIB 10657) and E (NCIB 10660) received from National Collection of Industrial Bacteria (NCIB), England and type D (ATCC 27517) from American Type Culture collection (ATCC), USA. These strains were used as reference strains.

3.2 Methods

3.2.1 Sampling

3.2.1.1 Fish/shrimp farms

Six farms located in and around Cochin were chosen for examination each varying from the others in type of farming practice, water source and differing geographical location. The farms were examined at monthly intervals for 6 months from October 1995 to March 1996.

Farms A and B are semi-intensive shrimp (*Penaeus monodon*) farms using back water and feeding dry commercially produced pelletized feed. Farm C is a pokali (paddy) field farming shrimp during December-March and June-September. In other aspects, it resembled farms A and B. Farms D and E are extensive farms doing polyculture with stocking fish and shrimp. There is no additional feeding. Farm F is an extensive shrimp (*Penaeus indicus*) farm with stocking and feeding commercially produced pelletized diet.

Water samples from six farms and feeder canals were collected aseptically in sterile 500 ml sampling bottles. Sediments were collected by means of a long handled metal scoop and transferred to sterile polythene bag and transported to the laboratory. Soil from farm and the immediate agriculture area and feed samples from shrimp farms were collected aseptically in sterile polythene bags and transported to the laboratory for estimation of microbiological parameters and for the detection of *C. botulinum*. Fish and shrimp samples were collected aseptically in sterile polythene bags. All the samples were transported immediately on ice to the laboratory and subjected to analysis for microbiological parameters and for *C. botulinum*.

Water samples were analysed for environmental parameters such as temperature, pH, dissolved oxygen and salinity.

3.2.1.2 Mud and bivalves from coastal waters

Mud samples collected from the coastal fish landing centres and coastal open water farms along the west and east coasts were transferred aseptically to sterile polythene bags and transported to the laboratory for the detection of *C. botulinum*.

Wild mussel samples collected from local seafood suppliers near Chellanam and Narakkal were transferred to sterile polythene bags and transported to the laboratory for

analysis. Farmed mussel (*Perna viridis*) samples and Oyster (*Crassostrea madrasensis*) samples were collected aseptically in sterile polythene bags from Calicut and from the long line culture trials at Andhakaranazhi and Narakkal of Central Marine Fisheries Research Institute, Cochin and transported to the laboratory under ice and analysed for *C. botulinum*.

3.2.1.3 Cultivable fish/shellfish and trash fish

Fish and shellfish listed by FAO (1995) as species used in aquaculture were selected for the study. Samples were collected aseptically in polythene bags either from department vessel operating off Cochin or from the retail outlets in and around Cochin and transported to the laboratory under ice and analysed for *C. botulinum*

Trash fish were procured from the department vessel operating off Cochin and from the retail outlets in and around Cochin and aseptically transferred into sterile polythene bags and transported immediately to the laboratory under ice and examined for the presence of *C. botulinum*.

3.2.1.4 Farmed shrimp for chilled storage

5kg of farmed shrimp (*Penaeus indicus*) were collected from a local shrimp farm and transported under ice to the laboratory within 1-2h Shrimp were beheaded, washed and iced in the ratio of 1:1 in an insulated ice box and maintained at 0°C for 12 days. Samples were withdrawn on 0 (fresh), 4, 8 and 12 day iced storage and qualitatively and quantitatively analysed for *C. botulinum*.

3.2.1.5 Frozen fish/shellfish

Fresh fish/shellfish were held at-23°C for one month in order to determine

whether or not low temperature storage has an adverse effect on the viability of *C. botulinum*.

Farmed shrimp (*Penaeus indicus*) and green mussel (*Perna viridis*) were collected as described in 3.2.1.4 and 3.2.1.2 and were washed after initial analysis, packed in polythene bag, quick frozen at -40°C and kept at frozen storage (-23°C) for 3-4 weeks and analysed for *C. botulinum*.

Fish/shellfish samples collected by the department vessel, off the south west coast of India, were washed, packed in polythene bags, quick frozen at -40°C onboard the vessel and stored at -18°C. They were transported to the laboratory and kept at frozen storage (-23°C) for 3-4 weeks and analysed for *C. botulinum*.

3.2.1.6 Cured fishery products

Cured fish/shellfish samples from the retail outlets, in and around Cochin, were aseptically collected in sterile polythene bags and transported to the laboratory for the estimation of chemical parameters such as salt content, moisture content and water activity and for the detection of *C. botulinum*.

3.2.2. Estimation of Environmental parameters

Fish/shrimp farms (A-F) were monitored at monthly intervals for environmental parameters such as temperature, pH, dissolved oxygen and salinity. Temperature of the farm water was monitored using a thermometer. The pH of the water sample was measured with a glass electrode using a pH meter (Systronics, Bombay). Dissolved oxygen and salinity were estimated according to the methods followed by Strickland and Parsons (1968).

3.2.3 Estimation of chemical parameters

Cured fish/shellfish samples procured from local retail outlets were analysed for sodium chloride and moisture contents. Moisture and sodium chloride contents were determined according to the methods followed by AOAC (1990). The water activity of cured fish samples were estimated using the water activity meter (Lufft a_w - Wert-Messer, Germany)

3.2.4 Estimation of microbiological parameters

Water samples collected aseptically from the farms (A-F) and feeder canals were serially diluted in sterile physiological saline and pour plated on PCA for total viable count (TPC) according to FDA (1995), on RCM agar for total anaerobic count (TAC) according to Barns (1985) and TSN agar for total clostridial count (TCC) according to Willis (1979).

Aseptically weighed 25g samples of mud into a sterile stomacher bag and added 225 ml physiological saline and homogenized for 1 min. in a stomacher (Seward Medical, London). All serial dilutions were prepared in sterile physiological saline and pour plated on PCA for TPC, RCM Agar for TAC and TSN agar for TCC.

Of farmed fish samples skin with muscle portions and intestine portions were analysed separately. The 3-4 fish were randomly selected for analysis. Skin with muscle portions were removed aseptically with sterile surgical scapel and forceps. Weighed 25g portions aseptically and emptied into sterile stomacher bag and added 225ml sterile physiological saline. The intestinal contents were aseptically removed and emptied into sterile stomacher bag The bags were then weighed and enough sterile physiological saline added to make a1:10 dilution. Samples were then homogenized for 1 min. in a

stomacher. All serial dilutions were prepared and pour plated for TPC on PCA, for TAC on RCM agar and for TCC on TSN agar.

Shell with muscle and intestine portions of shrimp were analysed separately. Whole shrimp samples were also analysed. In the case of shell with muscle portions and intestine portions, 10-12 shrimp samples were used for analysis as described above for fish samples.

Commercial pelleted feed samples collected from shrimp farms included starter A, B, grower and finisher feed. All feed samples were analysed for TPC, TAC, TCC, staphylococcus count, streptococcus count and coliform count. Feed samples were homogenized using a sterile mortar and pestle. Weighed 25g homogenized feed into a sterile stomacher bag and added 225ml sterile physiological saline, homogenized for 1 min in a stomacher. Serial dilutions were prepared and pour plated on PCA, RCM agar, and TSN agar and also on KF agar, for Streptococcus count. Samples were spread-plated on Baird-Parker (BP) agar for staphylococcus count. For coliform count, 10 ml portions were transferred to 3 double strength MC broth tubes and 1ml each to 3 tubes of single strength MC broth and 0.1ml each to 3 tubes of single strength MC broth

Plate count Agar plates were incubated aerobically at 30°C for 48h RCM agar plates and TSN agar plates were incubated anaerobically in Gaspak jar (Oxoid) at 30°C for 48h Baird parker agar plates and KF agar plates were incubated at 35°C for 24h and 48 h respectively. After incubation, colony forming units were counted. Readings obtained between 30 and 300 colonies on plates at the temperature of incubation were used for calculation of the bacterial populations and results recorded as CFU per unit of

sample. MC broth tubes were incubated at 35°C for 24 h After incubation for 24h tubes were examined for gas. Confirmed test was performed on positive (gassing) tubes only.

Clostridia encountered in farm water, sediment, farmed fish and shrimp were isolated from TSN agar plates and identified by the schemes proposed by Willis (1979; 1990), Mac Faddin (1980) and Sneath (1986).

3.2.5 Detection of *Clostridium botulinum*

Fish and shellfish samples obtained from onboard fishing vessels and from retail outlets in and around Cochin, farmed fish and shellfish, water, sediment, feed and soil from fish farms, chilled stored shrimp, frozen fish and shellfish and cured fishery products were examined for the presence of *C. botulinum* using a procedure followed by the US Food and Drug Administration (Solomon *et al.* 1995).

3.2.5.1. Enrichment

Weighed 3-5g skin with muscle portion of fish/shell with muscle portion of shrimp aseptically and inoculated into CMM (25 ml) after removing dissolved oxygen by steaming 10-15 min and cooling quickly without agitation. Intestinal contents of fish/shrimp were similarly inoculated and sterile paraffin oil was poured. Inoculated tubes were incubated at 30°C for 6 days. Whole fish/shrimp samples were also analysed.

Mussel and Oyster samples collected were shucked and homogenized. 5 g meat samples were subjected to enrichment in CMM for the detection of *C. botulinum* and incubated anaerobically as described above.

Water, sediment, soil and feed samples from fish/shrimp farms (A-F) were similarly analysed for *C. botulinum*. Inoculated 5ml amounts of farm water and feeder canal water into 25ml CMM. Weighed 5g each of sediment, soil and feed samples from

farms and transferred aseptically into 25ml CMM. Farmed fish/shrimp samples were analysed by separating skin/shell with muscle portions and intestine portions and inoculating separately into CMM as described above.

Sediment and shellfish samples collected from the west and east coasts were also analysed for *C. botulinum* as described above.

Shrimp samples withdrawn during chilled storage were similarly inoculated into CMM and incubated at 30°C for 6 days. The whole animal was used for the inoculation. After 12 days storage, 2-3 shrimp samples were withdrawn and packed in a vacuum packing machine (Sevana, India) and incubated for 6 days at 30°C.

Frozen and cured fish samples were examined for *C. botulinum* as previously described.

3.2.5.2. Detection of botulinum toxin

After incubation for 6 days at 30°C, cultures were centrifuged at 10000xg and 4°C for 20min (Remi, India) and each supernatant was adjusted to pH 6.2 with 1N HCl and frozen at -15°C overnight to eliminate non-specific mouse deaths (Baker *et al.* 1990). Supernatants were stored at -15°C until tested.

Toxicity of the supernatant was tested using a procedure followed by US Food and Drug Administration (Solomon *et al.* 1995). Diluted a portion of the supernatant to 1:5 in gelatin-phosphate buffer. Placed 1.8ml of the diluted supernatant in a tube and added 0.2ml of trypsin (Difco 1:250, 10% solution) and incubated at 37°C for 30 min. 1 ml of supernatant was placed in a tube and heated for 10min. in a boiling water bath and cooled. Tests for the presence of toxin in enrichment cultures were made by inoculation of pairs of webster mice intraperitoneally. Injected 0.5ml each of undiluted

supernatant, untreated and trypsinized diluted supernatant and heated supernatant using a 2ml syringe with a 25 gauge, 5/8 inch needle intraperitoneally into separate pairs of mice. Observed all mice periodically for 96 h for symptoms of botulism and death If after 48 h of observation, all mice except those receiving the heated preparation had died, repeated the toxicity test using higher dilutions of supernatant.

Typical botulism signs in mice were ruffling of fur, followed in sequence by laboured breathing, weakness of limbs and finally total paralysis with gasping for breath, followed by death due to respiratory failure.

The minimum lethal dose (MLD) is contained in the highest dilution killing both mice within 96 h From these data, the number of MLD/ml was calculated.

3.2.5.3 Typing of toxin

Rehydrated monovalent *C. botulinum* antitoxins A-F with sterile physiological saline to contain international unit (IU) per 1.0 ml

When the inoculated mice died with typical botulism symptoms, toxin neutralization tests were performed using the toxin preparation that gave the higher MLD, either untreated or trypsinized. Equal volumes of the sample diluted and each of antitoxin types A through F at potencies of 1.0 IU/ml were mixed together incubated at room temperature for 30min. and inoculated intraperitoneally into mice. When *C. botulinum* toxin was present, only the mice receiving the toxin neutralized with specific monovalent antitoxin specifically survived and the types present were identified.

3.2.6 Estimation of *Clostridium botulinum* numbers

Quantitative examinations of farm water, sediment, farmed fish/shrimp, farmed mussel, chilled stored shrimp and frozen shellfish were made to estimate the number of

C. botulinum present using the procedure followed by Cann and Taylor (1984) and Baker *et al.* (1990).

The 4 x 3 Most Probable Number (MPN) technique was used to estimate the number of *C. botulinum* spores in the samples analysed. Water samples of 10.0, 1.0, 0.1 and 0.01ml were inoculated into CMM overlayered with sterile paraffin oil and incubated at 30°C for 6 days. Tubes showing turbidity and gas production were tested for the presence of botulinum toxin.

Sediment and fish/shrimp-skin/shell with muscle and intestine samples of 1.0g, 0.1g, 0.01g and 0.001g were placed in CMM and incubated anaerobically at 30°C for 6 days and tested for the presence of toxin.

Homogenized mussel/oyster meat samples of 1g, 0.1g, 0.01g and 0.001g were inoculated into CMM by preparing appropriate dilution and incubated at 30°C for 6 days and tested for the presence of toxin as described in 3.2.5.2 and *C. botulinum* count was made using MPN tables found in FDA Bacteriological Analytical Manual (1995).

3.2.7 Statistical analysis

In order to find out the relationship between the environmental parameters, microbiological parameters and *C. botulinum* count of water, mud and farmed fish/ shrimp, their values were subjected to statistical analysis in a computer for the estimation of correlation coefficient 'r'. The significance 'p' of correlation coefficient 'r' of different parameters such as temperature, pH, salinity and dissolved oxygen were tested at 1% and 5% levels.

3.2.8 Isolation and characterization of *Clostridium botulinum*

Isolation and characterization of *C. botulinum* were attempted from the positive enrichment cultures of farm water, sediment, farmed fish, shrimp, mussel and fishery products using a procedure followed by Willis (1979) and Solomon *et al.* (1995). Reference strains of *C. botulinum* type D (ATCC 27517) and E (NCIB 10660) were used for comparative purposes.

3.2.8.1 Pretreatment

Recovery of toxigenic *C. botulinum* was attempted from toxic CMM enrichment cultures. To effect isolation, 2 ml each of enrichment culture sediment was transferred to two sterile test tubes. 2 ml of filter sterilized absolute ethyl alcohol was added to 1 tube and held at room temperature for 1 h with occasional shaking. The other tube was heated at 80°C for 10 min (Lalitha and Surendran 1993). After pretreatment, enrichment cultures were streaked on to predried plates of TSGYA and incubated anaerobically for 48-72h at 30°C. *C. botulinum* type D and E reference strains grown in CMM were similarly streaked on to predried plates of TSGYA and incubated anaerobically in Gaspak jar (Oxoid).

3.2.8.2 Selection and identification of *Clostridium botulinum* colonies

Colonies exhibiting surface irridescence covering both the zone of precipitation and the halo of clearing around the colony as seen by reflected light were picked up into tubes of CMM. The luster zone is usually referred to as pearly layer. The toxicity of each isolate was tested after incubation for 3 days at 30°C as described in 3.2.5.2. Those proved toxic were streaked again on TSGYA plates for confirmation of purity. The pure cultures were type identified by toxin neutralization test described in 3.2.5.3.

3.2.8.3 Characterization of *Clostridium botulinum*

Mrophological and biochemical characteristics of *C. botulinum* isolates were identified following the identification scheme of Willis (1979; 1990), Mac Faddin (1980) and Sneath (1986). Standard microbiological methods were used to interpret the tests (Willis 1979; 1990; Sneath 1986).

Morphological characteristics like shape, Gram reaction, presence or absence of spores and motility were determined. Biochemical characteristics like catalase production, gelatin liquefaction, hydrogen sulphide production, esculin hydrolysis, lecithinase production, lipase production, nitrate reduction, digestion of complex proteins, milk proteolysis and fermentation of sugars - glucose, maltose, lactose, sucrose, salicin, raffinose, fructose, galactose, mannose and melibiose were tested.

3.2.9 Sensitivity of fish to *Clostridium botulinum* toxin

3.2.9.1 Preparation of *C. botulinum* toxins

Pure strains of *C. botulinum* type A (53A), B (90B), C (18C), D (2693 D) and type E reference strain (NCIB 10660) were grown in CMM for 24h 1ml portion of 24h culture was inoculated into 25ml TYG broth whcih was subsequently incubated anaerobically at 30°C for 10-12d. The crude toxin was harvested by pouring the entire contents of the tube into centrifuge tubes and centrifuged for 20min at 10000 x g and 4°C in a refrigerated centrifuge (Remi, India). The supernatant (toxin) ranging in pH 6.7-7.0 was utilized on the same day as harvested after filtration using 0.2μ Millipore filter. The toxins were type identified as described in 3.2.5.3.

3.2.9.2. Fish

100-150 Tilapia (*Oreochromis mossambicus*) juveniles (8-10cm long) were obtained from a fish farm, in and around Cochin. The fish were held in 200 l tanks with a static water system until regular behaviour was established. Fish were fed with pelleted diet prepared in the laboratory once per day. The water was changed every alternate day. Aeration was supplied continuously by air stones. The sensitivity of fish to botulinum toxin was determined at 30°C in 50 l tanks. Three fish were put in each tank. The fish were acclimated for 3-4 days prior to testing

3.2.9.3 Sensitivity tests in fish

Tilapia were tested for sensitivity to different concentrations of *C. botulinum* types A-E toxin by the Intraperitoneal (IP) route. Serial dilutions of toxins were prepared in gelatin phosphate buffer and 0.5ml of each dilution was inoculated into each fish. Type E toxin was trypsinized before toxicity test as described in 3.2.5.2. The same concentration of toxin used for fish experiments were also inoculated into mice as described in 3.2.5.2. The lethal dose for fish was based upon the mouse IP lethal dose (MLD). A series of 3 fish were each inoculated IP with 1000, 100, 10, 1 and 0.5 mouse lethal doses (MLD) of toxin. One control fish was also held.

The clinical signs and other behaviour in fish were observed over a period of 7 days and mortalities were removed on a daily basis. The pathogenicity of botulinum toxin was confirmed by neutralizing the toxin with specific monovalent toxins as described in 3.2.5.3 and inoculating the fish. Those fish were survived which received toxin neutralized with specific antitoxin.

3.2.10 Effect of temperature on the stability of *Clostridium botulinum* toxin

3.2.10.1 Preparation of *Clostridium botulinum* toxins

Botulinum toxins of type A (53A), B (90B), C (18C), D (2693D) and type E reference strain (NCIB 10660) were prepared as described in 3.2.9.1. The initial amount of toxin present in the supernatent was determined by mouse assay as described in 3.2.5.2 and toxin type was confirmed by toxin neutralization test described in 3.2.5.3.

3.2.10.2 Studies on the stability of botulinum toxin to freezing

The toxins of *C. botulinum* types A, B, C, D and E were placed in test tubes in 5 ml amounts. 1 ml toxin of each type was diluted to 1:10, 1:100, 1:1000 and 1:10000 in GP buffer (pH 6.2). The amount of toxin in each dilution and also in the stock solution was estimated. The tubes were kept at -40°C for freezing After freezing, the tubes were stored at -15°C for 180 days and examined after 90 and 180 days for the inactivation of toxin by mouse assay as described in 3.2.5.2.

3.2.10.3 Studies on the thermal inactivation of botulinum toxins

To determine if storage at 30°C, 37°C and 40°C inactivates botulinum toxin, 2 ml amounts of *C. botulinum* type A (53A) and D (2693D) toxins were placed in small tubes and subsequently incubated at 30°C for 30 days, 37°C for 14 days and 40°C for 14 days and tested for any reduction in toxicity by mouse assay as described in 3.2.5.2.

To study the thermal inactivation of botulinum toxins of type A and D, 2 ml amounts of toxins were placed into small test tubes and given heat treatment using a procedure followed by Palumbo *et al.* (1995) and Grant *et al.* (1996). Each toxin was heat treated separately as follows: a glass tube (15 mm OD with 60mm height) containing 2 ml toxin was stoppered and secured in a metal rack along with a second

control tube containing 2 ml of TYG medium (control) without toxin into which a thermometer was inserted to monitor the temperature of the TYG medium during heating The rack was completely immersed in a water bath operating at temperature 50°C. The rack containing the tubes were agitated during the 90 sec. come up time but not during subsequent heating Timing began when the temperature of the control vial reached the experimental temperature. At intervals, appropriate for the temperature, vials were removed from the heating bath and cooled immediately in a slush ice bath for toxicity test. The same procedure was repeated for heat treatment at 60°, 70°, 80° and 100°C. Each heat treatment was duplicated.

After each heat treatment, a sub sample of toxin was subjected to the toxicity test for toxin estimation as described in 3.2.5.2. The types A and D toxins were again subjected to the above heat treatment by changing the heating menstruum. Sterile mullet tissue homogenate was used as the heating menstruum. Appropriate aliquots of the standard suspensions of toxins were added to 5 ml of fish tissue homogenate to yield inoculated mullet tissue containing Ca 10^3 MLD/ml for use in the heat inactivation trials as described above and the reduction in toxicity was estimated as described in 3.2.5.2.

3.2.11 Studies on growth characteristics of *Clostridium botulinum*

3.2.11.1 Influence of temperature

3.2.11.1.1 Influence of temperature on growth and toxin production

3.2.11.1.1.1 *Clostridium botulinum* strains used

Clostridium botulinum type A isolate 53A, reference strain NCIB 10640, type B isolate 90B, reference strain NCIB 10657, type C isolates 18C, 275C, type D isolates

2693D, 2131D reference strain ATCC 27517 and type E reference strain NCIB 10660 were used. They were maintained in CMM. *C. botulinum* strains were checked for purity by streaking onto TSGYA and single colonies were picked into CMM.

3.2.11.1.1.2 Preparation of spore suspensions

Spores of 10 strains were produced in CMM. After incubation at 30°C for 1-2 weeks, spores were separated using a procedure followed by Siegel and Metzger (1979) by centrifuging for 20min at 10000 x g and 4°C in a refrigerated centrifuge (Remi, India) and washed three times with sterile distilled water and resuspended in 50% ethanol for 1h to kill the vegetative cells (Tsang *et al.* 1985; Jensen *et al.* 1987), mixed and recentrifuged and stored in aqueous suspension at 1-4°C. The number of viable spores in the suspensions were determined by plating serial decimal dilutions on TSGYA and incubating at 30°C anaerobically in Gaspak jar (Oxoid) for 72h Plate count data were used to calculate the concentration of each spore suspension for use in subsequent studies.

3.2.11.1.1.3 Studies on the growth of *C. botulinum* at different temperatures

CMM was steamed for 20 min. and cooled rapidly before use. The appropriate dilution of the spore inoculum were made to obtain a concentration of Ca 10^5-10^6 spores/ml of C. b otulinum types A, B, C and D and for type E 10^3 spores/ml 1ml aliquot of each spore dilution thus obtained was inoculated into CMM. The CMM tubes were heated cooled and overlayered with 10-15 ml of presterilized paraffin oil to enhance anaerobiosis. The tubes were incubated at the required temperature (30°C, 15°C, 10°C and 4°C) for 24h before inoculation and were inoculated and incubated at the required temperature immediately. Five replications were performed for each temperature and for each strain. Controls (CMM without inoculation) were also used.

Examinations for growth (Gas and turbidity) were made daily for the first ten days of incubation and afterwards on every week upto 6 weeks. The tubes showing growth were assayed for toxin.

3.2.11.1.1.4 Detection of botulinum toxin

2 ml of the liquid portion of CMM was mixed with equal volume of GP buffer and centrifuged 20min at 10000 x g and 4°C. The pH of the supernatant was adjusted to 6.2. Supernatant was frozen overnight. Prior to injection, 0.2ml of fresh 10% trypsin solution was added to 1.8ml of the supernatant containing type E toxin and incubated at 37°C for 30min. The bioassay procedure was as described in 3.2.5.2. The controls were also injected into mice to eliminate the possibility of CMM giving false results for botulinum toxin and also to assure that the media were sterile. If death occurred, neutralization tests were made as described in 3.2.5.3.

3.2.11.1.2 Effect of temperature and substrate on toxin production

3.2.11.1.2.1 Clostridium *botulinum* strain

Clostridium botulinum type B strain (90B) was used. The culture was maintained and checked for purity as described in 3.2.11.1.1.1. A 24 h CMM culture was employed in studies on kinetics of toxin production.

3.2.11.1.2.2. Studies on kinetics of toxin production

The three media, CMM, TSGYB and TPGYB were deoxygenated rapidly cooled and immediately inoculated with 1 ml of a log phase culture and overlayered with sterile paraffin oil. The flasks were incubated at 30°C and 37°C anaerobically for 96 h Samples were withdrawn after 0, 24, 42, 48, 72 and 96 h and examined for growth and toxin production.

3.2.11.1.2.3 C. *botulinum* count

The initial *C. botulinum* count of the log phase culture was determined by the method described in 3.2.11.1.1.2 for spore count.

The number of viable cells after 24 h, 42 h, 48 h 72 h and 96 h in the three media inoculated were also determined.

3.2.11.1.2.4 Botulinum toxin assay

Botulinum toxin present in samples withdrawn after 0, 24h, 42h, 48h, 72h and 96h was assayed. Cell free culture fluids were obtained by centrifugation at 10000 x g and 4°C for 20min, using the procedure followed by Siegel and Metzger (1979). Due to the lability of toxin, certain precautions were taken during manipulation of the samples to be assayed. Dilutions were made in cold sterile GP buffer. Toxicity was assayed as described in 3.2.5.2 and toxin titers were calculated.

3.2.11.2 Influence of pH

3.2.11.2.1 C. *botulinum* strains used

The following strains of *C. botulinum* were used. Type A strains 53A and 131A isolated from fish and mussel, reference strain NCIB 10640, type B strain 90B isolated from fish, type C strains 18C and 275C isolated from fish, type D strains 2693 D isolated from sediment, 2131D isolated from cured fish, reference strain ATCC 27517 and type E reference strain NCIB 10660. The cultures were maintained and checked for purity as described in 3.2.11.1.1.1.

3.2.11.2.2. Preparation of spore suspensions

Spores of ten strains were produced and counted as described in 3.2.11.1.1.2.

3.2.11.2.3 Studies on the growth of *C. botulinum* at different pHs

CMM was adjusted from pH 6.0 to 4.6 at 0.2 pH unit intervals by the addition of dilute HCl using a pH meter (Systronics, Bombay). CMM (pH 7.0) was also included in the study. For each pH, 5 replications were performed for each strain. CMM tubes were deoxygenated, cooled and immediately inoculated with 1ml of unheated spore suspension equivalent to 0.1-1 million viable spores of types A, B, C and D and 1 thousand viable spores of type E separately. Each tube was sealed with sterile paraffin oil and incubated at 30°C. Examinations for growth were made daily for the first two weeks and afterwards at intervals upto 5 weeks. Toxicity was tested controls (CMM without inoculation) were also used to assure that the media were sterile and the pH did not change during the 5 week period.

3.2.11.2.4 Detection of botulinum toxin

Samples withdrawn from CMM tubes were assayed for toxin as described in 3.2.11.1.1.4.

3.2.11.3 Influence of water activity

3.2.11.3.1 *Clostridium botulinum* strains used

The strains described in 3.2.11.2.1 and type B reference strain (NCIB 10657) were used. The cultures were maintained and checked for purity as described in 3.2.11.1.1.1.

3.2.11.3.2 Preparation of spore suspensions

Spores of 11 strains of *C. botulinum* were produced and counted as described in 3.2.11.1.1.2.

3.2.11.3.3 Studies on the growth of *C. botulinum* at different water activities (a_w)

CMM (pH 7.0) was used for the water activity (a_w) studies with the addition of

various concentrations of sodium chloride (NaCl). The CMM (0, 3, 4, 5, 6, 8, 10 and 12% NaCl) were prepared on a weight volume basis. The a_w was determined using the water activity meter (Lufft a_w Wert-Messer, Germany). For each salt level, five replications were performed for each strain. CMM tubes were deoxygenated, cooled and immediately inoculated with 1ml of unheated spore suspension equivalent to 0.1-1 million viable spores of types A, B, C and D and 1 thousand spores of type E separately. Controls (CMM without inoculation) were used to assure that the media were sterile. Each tube was sealed with presterilized paraffin oil and incubated at 30°C. Examinations for growth (gas and turbidity) were made daily upto 14 days and after that at weekly intervals for a period of 5 weeks and toxicity were tested.

3.2.11.3.4 Detection of botulinum toxin

Samples from tubes showing growth were withdrawn and tested for the presence of toxin as described in 3.2.11.1.1.4.

3.2.11.4 Effect of gaseous atmosphere

3.2.11.4.1 *Clostridium botulinum* strains used

The following strains of *C. botulinum* were used. Type A strain 53A, type B 90B, type C 18C, type D 2693D and reference strain (ATCC 27517) and type E reference strain (NCIB 10660). The cultures were maintained in CMM and checked for purity as described in 3.2.11.1.1.1 and grown in CMM.

3.2.11.4.2 Preparation of spore suspensions

Spores of six strains were produced and counted as described in 3.2.11.1.1.2.

3.2.11.4.3 Preparation and inoculation of fish/shrimp tissue homogenates for vacuum pack studies

15 fresh mullet (*Mugil cephalus*) were purchased from local retail market in Cochin and transported on ice to the laboratory. Each fish was rinsed in tap water, drained and filleted. Fillets were ground in a mechanical blender and divided into 10g portions in 25 x 150mm tubes and autoclaved at 121°C for 15 min.

Fresh shrimp (*Penaeus indicus*) were procured from local retail market in Cochin and brought to the laboratory under ice. Shrimp were washed, deheaded, peeled, deveined and ground in a mechanical blender. 10g portions were placed in 25 x 150mm tubes and autoclaved for 15 min at 121°C.

The 10g portions of sterile mullet tissue and shrimp tissue homogenates were transferred aseptically to sterile pouches (12 micron plain polyester laminated with 150 gauge low density polythene pouch). The Mullet and Shrimp tissue homogenates were inoculated with 0.1ml of spore inoculum equivalent to 0.1-1 million viable spores of types A, B, C, D and 1 thousand viable spores of type E separately. To simulate the naturally occurring situation, none of the inocula was heat shocked. Control samples were inoculated with 0.1 ml of sterile physiological saline in a like manner. The pouches were sealed in a vacuum pack machine (Sevana, India).

Vacuum packed pouches were stored at 30°C for 3 days, at 15°C for 10 days, at 10° and 4°C for 6 weeks. Samples stored at 30°C were examined daily for growth and toxicity. Samples stored at 15°C were examined daily for first 5 days and after that on 6, 8 day and assayed for toxicity. For the 10°C storage, samples were assayed for toxin on 1, 2, 5, 8 and 10 day. Samples held at 4°C were examined at 3 day intervals and assayed

for toxin. For each storage temperature three replications of mullet tissue and shrimp tissue homogenates were performed for each strain.

In order to study the significance of storage temperature on growth and toxin production 1-2 pouches stored at 10°C and 4°C were transferred after 40 days to 30°C for 5 days and tested for toxin production.

3.2.11.4.4. Detection of toxin

Samples showing growth were extracted overnight with 3 ml of gelatin phosphate buffer. The mixture was centrifuged at 10000 x g and 4°C for 20 min and the supernatant was collected. If the pH of the supernatant containing type E toxin was equal to or more than 7.0, it was adjusted to pH 6.2 and trypsinized. Toxicity test, estimation of toxin, toxin neutralization tests were performed as described in 3.2.5.2 and 3.2.5.3.

3.2.11.5 Combination Effects

3.2.11.5.1 Combined effect of temperature and pH

3.2.11.5.1.1 *Clostridium botulinum* strains

The following strains were used : type A strain 53A, type C strain 18C, type D strain 2131D and type E reference strain (NCIB 10660). The cultures were maintained and checked for purity as described in 3.2.11.1.1.1.

3.2.11.5.1.2 Preparation of spore suspensions

Spores of four strains were produced and counted as described in 3.2.11.1.1.2

3.2.11.5.1.3 Studies on the combined effect of temperature and pH

CMM was adjusted to pH 7.0, 6.0, 5.5, 5.0 and 4.5 as described in 3.2.11.2.3. Five replications were performed for each pH and each spore inoculum. Inoculation of the tubes were made as described in 3.2.11.2.3. Each tube was overlayered with sterile

paraffin oil. Tubes were incubated at 30°C, 15°C, 10°C and 4°C. For the 30°C storage, tubes were examined daily for 16 days. Tubes stored at 15°C were examined daily for the first one week and afterwards on every four days. For the 10°C and 4°C storage, tubes were examined on every three days and tubes showing growth (gas and turbidity) were tested for the presence of toxin.

3.2.11.5.1.4 Detection of botulinum toxin

Tubes showing growth were withdrawn and toxicity was tested as described in 3.2.11.1.2.4

3.2.11.5.2 Effect of incubation temperature on salt tolerance of *C. botulinum*

3.2.11.5.2.1 *Clostridium botulinum* strains used

The following strains were used. Type A strain 53A, type C strain 18C, type D strain 2693D and type E reference strain (NCIB 10660). The cultures were maintained and checked for purity as described in 3.2.11.1.1.1 and grown in CMM.

3.2.11.5.2.2 Preparation of spore suspensions

Spores of four strains were produced and counted as described in 3.2.11.1.1.2.

3.2.11.5.2.3 Studies on the effect of incubation temperature on salt tolerance

CMM was used with the addition of sodium chloride at levels of 0, 3, 5, 8 and 10%. The media were prepared on a weight volume basis. The water activity of the media was determined by the water activity meter (Lufft a_w Wert Messer, Germany).

For each salt level, five replications were performed for each temperature and strain. Tubes were inoculated as described in 3.2.11.3. Controls (CMM without inoculation) were used to assure that the media were sterile. Each tube was sealed with sterile paraffin oil and incubated at 30°C, 15°C, 10°C and 4°C. When incubation at low

temperatures was desired, those tubes were tempered in either cold water or ice water before inoculation. For the 30°C storage, tubes were examined daily for two weeks and after that every alternate day. Tubes held at 15°C were examined daily for first one week and afterwards on every two days. For the 10° and 4°C storage, tubes were examined on every three days upto 5 weeks. Tubes showing growth (gas and turbidity) were tested for toxin production.

3.2.11.5.2.4 Detection of botulinum toxin

Tubes showing growth were withdrawn and toxicity test was performed as described in 3.2.11.1.1.4

3.2.11.5.3 Inhibitory effect of combinations of pH, sodium chloride and incubation temperature

3.2.11.5.3.1 Clostridium *botulinum* strains

Clostridium botulinum strains described in 3.2.11.5.2.1 were used in the study. The cultures were maintained and checked for purity as described in 3.2.11.1.1.1.

3.2.11.5.3.2 Preparation of spore suspension

Spores of four strains of *C. botulinum* were produced and counted as described in 3.2.11.1.1.2.

3.2.11.5.3.3 Studies on the combined effect of pH, sodium chloride and Incubation temperature

CMM with sodium chloride levels 0, 3, and 5% were prepared on a weight volume basis. In one set of tubes (0%, 3%, 5% salt) the pH was adjusted to 7.0 and in the other set, pH was adjusted to 5.5. The water activity of the two sets of media were determined by the water activity meter (Lufft a_w Wert-Messer, Germany).

For each salt level adjusted to pH 7.0, five replications were performed for each temperature and strain. Similarly five replications were performed for each salt level at pH 5.5 for each temperature and strain. Inoculated tubes in each set were incubated at 30°C and 15°C for a period of 6 weeks. Tubes were inoculated as described in 3.2.11.2.3 and overlayered with sterile paraffin oil. Controls (CMM without inoculation) were used to assure that the media were sterile. Examination of the tubes were performed as described in 3.2.11.5.2.3 and toxicity was tested.

3.2.11.5.3.4 Detection of botulinum toxin

Tubes showing growth were withdrawn and tested for toxin production as described in 3.2.11.1.1.4.

4. RESULTS AND DISCUSSION

4.1 Clostridium *botulinum* in farmed and wild fish/shellfish

4.1.1 Distribution of *Clostridium botulinum* in fish/shrimp farms

Results of the microbial counts of water, mud, feed, fish and shrimp samples from six farms (A-F) located in and around Cochin, during farming operations for the period October 1995 to March 1996 are shown in Figs. 1-6. The changes in the environmental parameters are given in table 1.

The total viable plate counts (TPC) of the water samples ranged from 10^3 to 10^4 CFU/ml. and that of mud were in the ranges of 10^4 to 10^6 CFU/g. The Total Anaerobic Count (TAC) of pond water and mud varied between 10^1 to 10^2 CFU/ml and 10^2 to 10^4 CFU/g respectively. The total clostridial count (TCC) of pond water ranged from 10 to 10^2 CFU/ml. and that of mud varied between 10^1 to 10^3 CFU/g. In general, all the 3 microbial counts were high in pond mud compared to pond water.

In farmed fish, the TPC of surface samples varied between 10^4-10^6 CFU/g while the TAC ranged from 10^2 to 10^4 CFU/g and the clostridial count varied between 10^1 to 10^3 CFU/g. The microbial counts of surface samples of fish were in the same range as that of mud samples. The TPC, TAC and TCC of intestine sample of fish varied between 10^5 to 10^7 CFU/g, 10^3-10^5 CFU/g and 10^2-10^4CFU/g respectively. The microbial counts were high in the intestine samples of fish compared to fish surface.

In the whole shrimp, TPC, TAC and TCC were in the range of 10^4-10^5 CFU/g, 10^3-10^4 CFU/g and 10^2-10^3 CFU/g respectively. In shrimp, the TPC, TAC and TCC of shell with muscle portion ranged from 10^4 to 10^5 CFU/g, 10^2-10^4 CFU/g and 10^1-10^3 CFU/g respectively while that of intestine varied between 10^7-10^8 CFU/g, 10^4-10^5 CFU/g

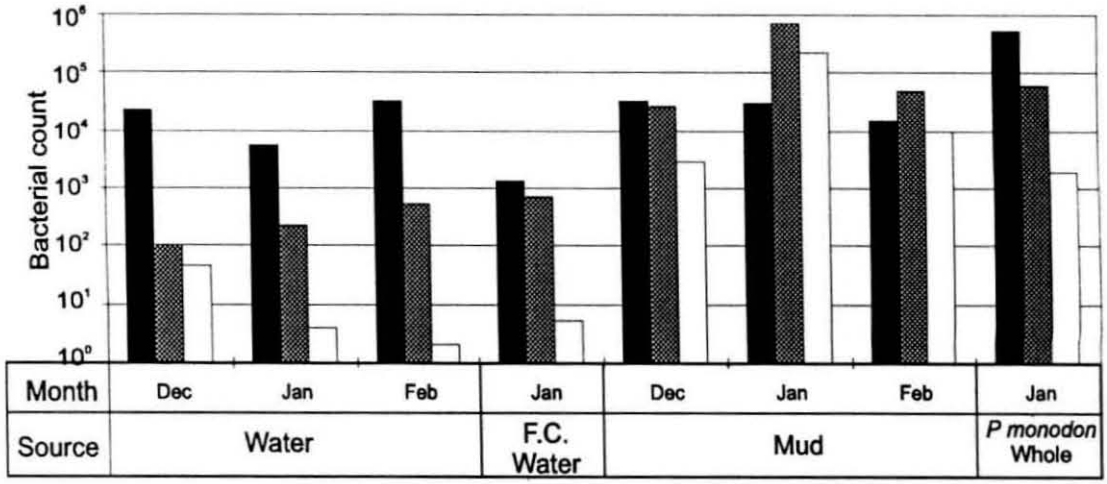

Fig. 1 .Microbial count of Water, Mud and Shrimp from Farm A

Count of water in CFU / ml. Count of mud and shrimp in CFU/g

■Total Plate Count ▩Total Anaerobic Count □ Total Clostridial Count

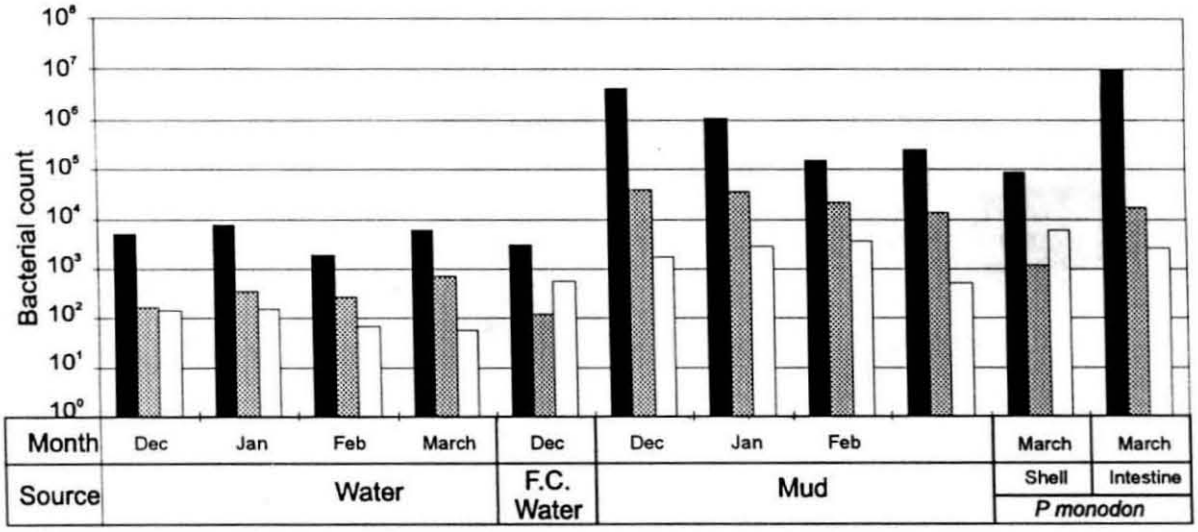

Fig. 2 .Microbial count of Water, Mud and Shrimp from Farm B

Count of water in CFU / ml. Count of mud and shrimp in CFU/g

■Total Plate Count ▩Total Anaerobic Count □ Total Clostridial Count

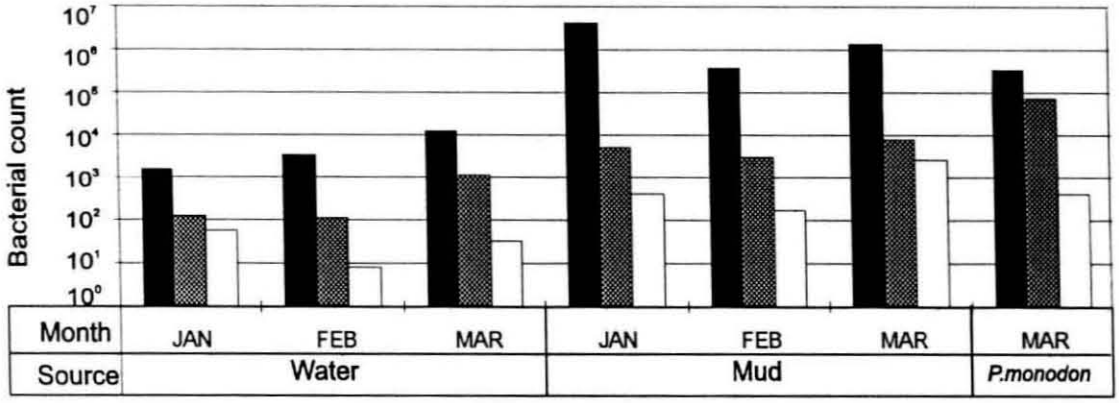

Fig. 3. Microbial count of Water, Mud and Shrimp from Farm C

Count of water in CFU / ml. Count of mud and shrimp in CFU/g

■Total Plate Count ▩Total Anaerobic Count □ Total Clostridial Count

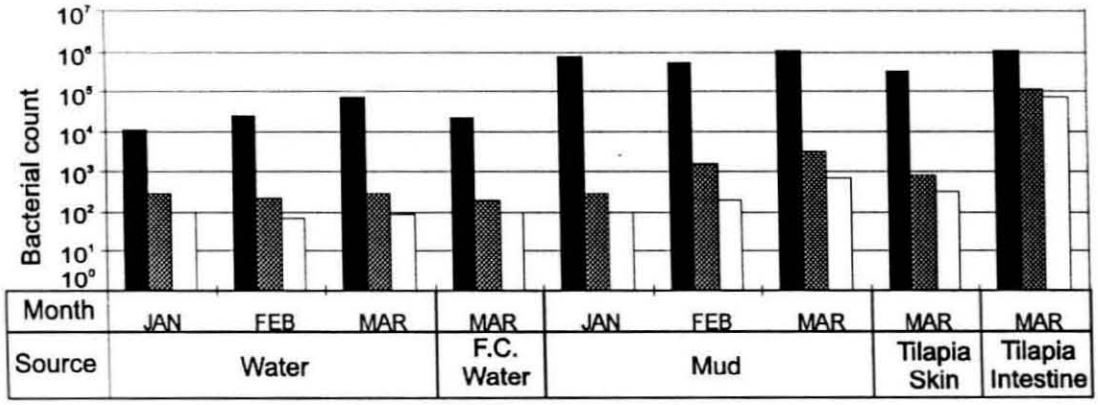

Fig. 4. Microbial count of Water, Mud and Fish from Farm D

Count of water in CFU / ml. Count of mud and shrimp in CFU/g

■Total Plate Count ▩Total Anaerobic Count □ Total Clostridial Count

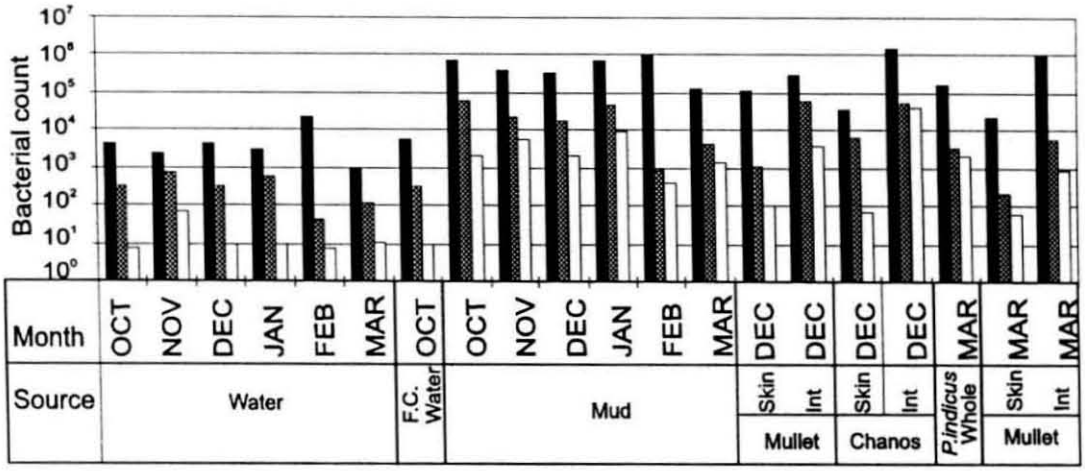

Fig. 5. Microbial count of Water, Mud, Fish and Shrimp from Farm E

Count of water in CFU / ml. Count of mud and shrimp in CFU/g

■Total Plate Count ▩Total Anaerobic Count □ Total Clostridial Count

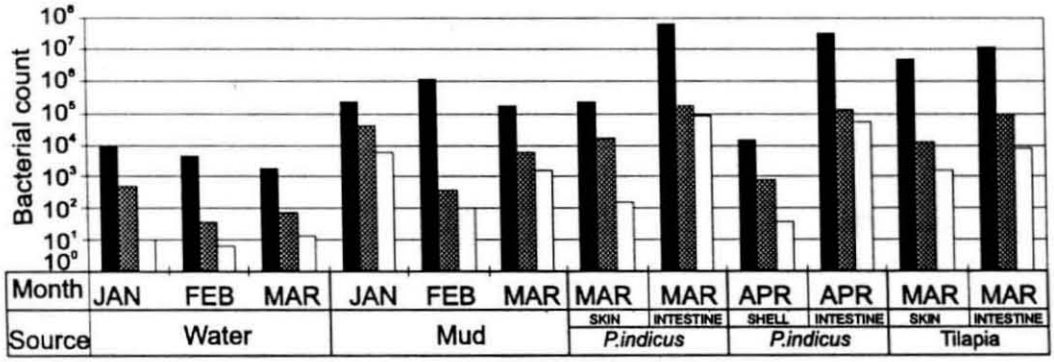

Fig. 6. Microbial count of Water, Mud, Shrimp and Fish from Farm F

Count of water in CFU / ml. Count of mud and shrimp in CFU/g

■Total Plate Count ▩Total Anaerobic Count □ Total Clostridial Count

and 10^2-10^4 CFU/g respectively. As in the case of fish, all the three counts were higher in the intestine than in the surface samples of shrimp. Intestine samples of shrimp harboured high microbial counts compared to mud samples and fish intestine samples.

The microbial counts of the commercial pelleted feed samples collected from the shrimp farms were also determined. The starter, grower and finisher feeds analysed showed that the TPC varied between 10^4-10^6 CFU/g, the TAC ranged from 10^2-10^4 CFU/g and the TCC varied between 10^2-10^3 CFU/g (Fig. 7). Streptococcal count of the Grower feed was 10^3 CFU/g. Staphylococci and *Escherichia coli* were not present. While Bacillus and yeast predominated in the total viable count, Clostridium and Eubacterium constituted the total anaerobes.

Clostridium botulinum was detected only in three (A, E, F) out of the six farms examined (Table 2 and 3). Of the 34 water samples examined, only 6% were positive for *C. botulinum*. One of the three soil samples tested from the farm area harboured C. *botulinum* and type C toxin was detected in the sample. *C. botulinum* was found in 11 percent of the 28 mud samples from the six farms. Type C toxin was detected in two samples and type D in one. In farmed shrimp, contamination by *C. botulinum* was found in twenty percent (4/20) of the samples. While type D toxin was found in three samples, type C was present in one. The incidence of *C. botulinum* in farmed fish was 18 percent (2/11). Types C and D toxins were detected in the positive samples. *C. botulinum* could not be detected in commercial pelleted feed samples collected from the farms.

The counts of *C. botulinum* ranged from 0-4 per 100 ml. in water samples and 0-11/g in the mud samples (Table-4). In shrimp, the count was in the range of 0-150/100g and in the intestine samples of fish, the count ranged from 0-70/100g. The

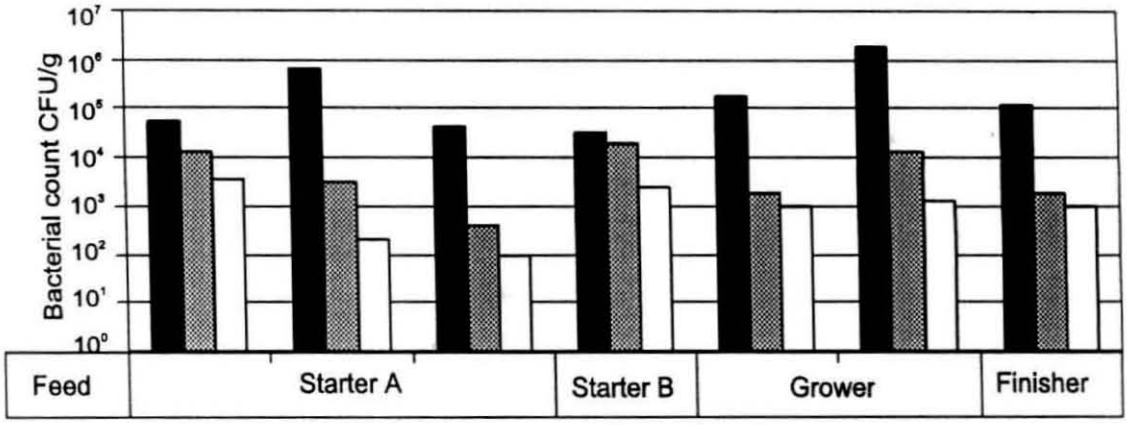

Fig. 7 .Microbial count of Commercial Shrimp Feed

■Total Plate Count ▩Total Anaerobic Count □ Total Clostridial Count

toxin titres were low (10-20 MLD/ml) in the enrichment cultures of mud, shrimp and fish in Robertson's cooked meat medium (CMM).

Table 1: Environmental characteristics of the fish and shrimp farms in and around Cochin

Farm/Species Farmed	Temp. °C	pH	D.O. (mg/l)	Sal. (ppt)
A/*P. monodon*	30-31	8.40-9.66	5.4-6.8	15.12-19.52
B/*P. monodon*	28-31	6.99-9.93	4.4-6.2	2.73-23.31
C/*P. monodon*	30-32	6.81-7.88	6.0-8.0	16.64-23.31
D/ *O.mossambicus*	30-31.5	7.10-7.60	5.6-6.66	8.3-10.53
E/*Chanos*, Mullet, *P. indicus*	28-31.5	7.60-8.40	5.6-10.8	6.89-30.7
F/*P. indicus*	30-33	7.60-8.15	3.6-6.2	19.5-24.58

The environmental parameters in the farms (A-F) remained within the acceptable range. However, wide fluctuations in pH were observed in farms A, B and C. The lower pH limit of 6.81 and 6.99 observed in farms B and C and the upper pH limit of 9.66 and 9.93 in farms A and B are not ideal for shrimp farming. Diurnal fluctuations of pH 8.0 to 9.6 have been reported from shrimp farms (Vijayakumaran and Paulraj 1997). No significant correlation was observed between the environmental parameters and the microbial counts of water, mud and shrimp/fish. There was statistically significant

positive correlation ($P < 0.05$) between the TPC of mud and TPC of water, TPC of mud and TPC of shrimp, TAC and TCC of mud and *C. botulinum* count. Matrix of correlation was constructed for pond water and mud separately in order to study the extent of interdependency of *C. botulinum* count in *Penaeus monodon* with environmental factors (Table 5 and 6). There was no significant correlation between *C. botulinum* count in *P. monodon* and that of water and environmental parameters at 5% level. But there was significant positive correlation between C. *botulinum* count in P. *monodon* and C. *botulinum* count in the mud ($P < 0.05$), *C. botulinum* count of mud and pH ($P < 0.10$) indicating that as the pH increases *C. botulinum* numbers of the mud increases and as the count of *C. botulinum* in the mud increases, *C. botulinum* count of *P. monodon* increases.

The TPC of pond water reported in the present study was within the ranges quoted in earlier studies (Acuff *et al.* 1984; Fonseka 1990; Nedoluha and Westhoff 1993; Surendran *et al.* 1994; Nayyarahamed *et al.* 1994; Sharmila *et al.* 1996). The TPC of pond mud observed in this study was also comparable to the levels of TPC reported by Sugita *et al.* (1989), Llobrera *et al.* (1990), Surendran *et al.* (1994) and Sharmila *et al.* (1996). The TPC of whole shrimp was within the range of reported values (10^4-10^5 CFU/g) of shrimp from tropical countries (Llobrera *et al.* 1990; Fonseka 1990). Bacterial counts of surface samples of farmed fish, 10^4-10^6 CFU/g, was within the ranges quoted in previous studies (Acuff *et al.* 1984; Nedoluha and Westhoff 1993). Bacterial counts of 10^5-10^8 CFU/g have been reported for the intestine samples of pond reared stripped Bass (Nedoluha and Westhoff 1993) and farmed freshwater fishes (Sugita *et al.* 1985; Sivakami *et al.* 1996). Similar counts were reported in the present study. The TPC of pelleted feed from shrimp farms observed in the present study was comparable to the

levels of TPC for pelleted feed reported earlier (Trust 1971; Sugita *et al.* 1989; Ogbondeminu *et al.* 1991). The numbers of faecal streptococci in pelleted feed reported in the study are high. Trust (1971) had reported streptococci count of 10^2-10^3 CFU/g in commercial fish diets. The higher bacterial load of the habitat, i.e. pond sediment, was reflected in the same trend in the TPC of shrimp by virtue of its bottom dwelling habit.

Sugita *et al.* (1989) reported total anaerobic count of 10^1-10^2 CFU/ml in water samples from culture ponds. Similar counts were observed in the present investigation. In contrast, Nedoluha and Westhoff (1993) reported anaerobic counts of 10^3-10^5 CFU/ml from water samples of freshwater ponds in Maryland. The TAC of mud from the farms in the study ranged from 10^2-10^4 CFU/g. Sugita *et al.* (1989) reported anaerobic counts of 10^2-10^7 CFU/g for sediments from a gold fish culture pond. Anaerobic counts of 10^2-10^5 CFU/g was reported for bottom muds from rivers and Bays in Japan (Takano *et al.*1984). The anaerobic counts of surface samples of farmed fish reported in this study was within the ranges quoted earlier (Nedoluha and Westhoff 1993). The anaerobic count from the intestine samples of farmed fish in the present study was in the range of 10^3-10^5 CFU/g. In contrast, Nedoluha and Westhoff (1993) reported anaerobic counts of 10^6-10^8 CFU/g from the intestine of pond reared stripped Bass. Sugita *et al.* (1985) reported anaerobic counts of 10^2 CFU/g from the intestine of Tilapia reared in tanks. For whole shrimp, anaerobic counts of 10^4 CFU/g recorded in the present study are within the range reported earlier (Wibowo *et al.* 1991). Anaerobic counts of 10^2-10^4CFU/g for pelleted feed were reported by Trust (1971) and Sugita *et al.* (1989). The counts in the present study ranged from 10^2-10^4 CFU/g.

Clostridia were detected at densities ranging from 10^1-10^2 CFU/ml in gold fish reared water (Sugita *et al.* 1989). Similar counts were noticed in the present study. The clostridial count of 10^6 CFU/g in sediments from gold fish culture pond, reported by Sugita *et al.* (1989) was high compared to the counts reported in the present study (10^1-10^3 CFU/g). The clostridial count of intestine samples of farmed fish varied between 10^2-10^4 CFU/g in the present study. Clostridia were detected in the intestine of marine fish at densities ranging from 10^2-10^4 CFU/g in Japan (Sugita *et al.* 1988) and 10^4-10^7 CFU/g in India (Lalitha *et al.* 1990). Unfortunately most of the data available are from the study of wild fish.

The species of clostridia isolated from the farm water, mud, shrimp and fish samples were characterised. The clostridia most frequently encountered in farm water were C. *perfringens, C. sardiniense, C. clostridioforme, C. fallax, and C.celatum* and C. *absonum*. The farm mud harboured clostridia such as *C. perfringens, C. novyi C. fallax, C. sardiniense, C.sporogenes, C.butyricum, C. felsineum, C.tertium* and *C. bifermentans.*

In farmed shrimp, *C.sardiniense, C.sporogenes* and *C.bifermentans* were encountered in addition to *C. perfringens* and *C. novyi.* In farmed fish, apart from C. *perfringens* and *C. novyi* the species most often recovered were *C. sardiniense, C. sporogenes, C. butyricum* and *C. bifermentens*, but *C. fallax, C. carnis* were also isolated. Davies (1969) isolated *C. perfringens, C. sordelli* and *C. bifermentans* from marine sediments. Matches and Liston (1974) reported that most of the mesophilic anaerobes from marine sediments of Puget Sound belonged to clostridial species *C. perfringens, C. bifermentans* and *C. novyi.* Smith (1975) isolated *C. subterminale,*

C. sporogenes, C. perfringens, C. bifermentans, C. indolis and *C. mangenoti* most frequently from soil samples. In the present study, several other species of clostridia were also encountered in addition to those observed by Davies (1969) and Matches and Liston (1974). The difference in the clostridial species found probably reflects a difference in the anaerobic flora of the sites examined. Shewan (1977) reported clostridium species such as *C. perfringens, C. sporogenes, C. tetani, C. tertium* and *C. botulinum* type E in the intestine of fish. Lalitha *et al.* (1990) isolated *C. perfringens* from fish and shrimp. The clostridium species reported in fish and shrimp in the present study were similar to the species encountered in the mud. Horsely (1973) reported that bacterial flora of fish is a reflection of its environment. The anaerobic flora of pelleted diet in the present study composed of clostridia as reported earlier (Sugita *et al.* 1989).

The level of contamination by *C. botulinum* was 33% in soil samples from the farm surroundings and type C was detected in the present study. Pasricha and Panja (1940) found *C. botulinum* type A in 50% of the soil samples from Calcutta. Hayashi *et al.* (1981) reported 20% of the soil samples from Indonesia to be contaminated with *C. botulinum,* with type D predominating followed by type C. In the Gulf of Thailand, types C and D predominated in sand samples. *C. botulinum* types C and D are the predominant types in soil samples from tropical areas (Hayashi *et al.* 1981; Tanasugarn 1979) whereas in the soil of Western United States, Brazil, Argentina and China type A predominated and type B was predominant in soils from eastern United States and U.K. (Smith 1990; Mc Clure *et al.* 1994). Hobbs (1976) attributed the regional variations in the incidence of botulism to the variable incidence of spores in local soils or offshore waters and substantiated the earlier view of Dolman that the primary source of this

organism was soil. Cann *et al.* (1975) found that the natural occurrence of *C. botulinum* in the soil of the area surrounding the farm is one of the factors which contribute to the contamination of fish in the farms. The predominance of types C and D in the farm environment and farmed species in the present study can be attributed to the predominance of these types in the soil surrounding the farm.

In the present investigation the incidence of *C. botulinum* in pond mud was 11% with a spore load of 0-11/g. Types C and D were detected in the positive samples. Burns and Williams (1975) detected *C. botulinum* type B in 20 percent of mud samples from Scottish farms. Cann and Taylor (1984) detected types B and E in British farms with a spore load of 0.1-0.7/g whereas in farms with fish botulism outbreak type E was detected in 60% of the mud samples with a spore load of 1-800/g. Huss (1980) detected C. *botulinum* types B and E in 77% of the sediment samples from artificial ponds in Denmark. In the United States, *C. botulinum* type E populations ranging from 23 to 240000 per g. were reported in sediments from trout ponds in which botulism outbreaks were occurring (Eklund *et al.* 1982; 1984). In farm water, *C. botulinum* was detected at a level of 4/100 ml. in the present study. Huss *et al.* (1974a) reported *C. botulinum* in the outlet waters of Danish trout farms at the level of 8.5/10l. Pond mud was shown to be an obvious source of contamination by *C. botulinum* in fish and shrimp farms in the present study as reported earlier (Bach *et al.* 1971; Burns and Williams 1975; Cann and Taylor 1982).

In farmed fish, the occurrence of *C. botulinum* was 18% in the present study. The viscera were shown to be a major reservoir of contamination. Cann *et al.* (1975) reported

that the rate of contamination by *C. botulinum* in whole fish was 9% and in viscera of fish it was 11% in British trout farms. The prevalence of *C. botulinum* was high in Danish and British trout farms (Huss *et al.* 1974a; Cann and Taylor 1982), fish farms in Scotland (Burns and Williams 1975) and cat fish farms in California (Baker *et al.* 1990). Baker *et al.* (1990) reported 75% of the farm raised cat fish in California and 25% of the farm raised cat fish in Mississippi harboured *C. botulinum* type A. *C. botulinum* was detected in none of the samples in a survey of aquacultured hybrid stripped Bass from Maryland (Nedoluha and Westhoff 1993). *Clostridium botulinum* types C and D were detected in the intestine samples of fish in the present study. Burns and Williams (1975) detected non-proteolytic types B in fish from Scottish farms. The occurrence of *C. botulinum* type C was reported most frequently in farmed trout from Great Britian. In the same study, four of the 7 known types of *C. botulinum* (B, C, E, F) were found in farmed trout (Cann *et al.* 1975). Cann *et al.* (1975) found type F was confined to Scotland and type E to England. Huss *et al.* (1974a) found C. *botulinum* types B, E and a mixture of types A and E in trouts from Danish farms. *C. botulinum* types prevalent in fish farms in the present study differ from that reported in Europe and United States (Huss *et al.* 1974; Burns and Williams 1975; Baker *et al.* 1990; Eklund *et al.* 1982).

The incidence of *C. botulinum* in shrimp in the present study was 20%. Most of the data available for shrimp are from wild species. Incidence of *C. botulinum* was 50% (3/6) in prawns from retail markets in Cochin, India, (Lalitha and Surendran 1992). Suhadi *et al.* (1981) reported incidence of *C. botulinum in* 2% of the commercial shrimp samples in Indonesia. In Japan, 8% of the shrimp samples from Osaka markets harboured

C. botulinum (Haq and Sakaguchi 1980). West (1989) reported low incidence of C. *botulinum* in shellfish.

Table-2: Incidence of *Clostridium botulinum* in the fish and shrimp farm environment

Farm	Type of sample tested	No. of samples tested for *C. botulinum*	No. of samples positive for *C. botulinum*	Type of toxin
A	Pond water	3	1	D
	Pond mud	3	2	C, D
	Soil from farm surroundings	1	1	C
	Feeder canal water	1	-	-
B	Pond water	4	-	-
	Pond mud	4	-	-
	Feeder canal water	1	-	-
C	Pond water	3	-	-
	Pond mud	3	-	-
D	Pond water	5	-	-
	Pond mud	5	-	-
	Soil from farm surroundings	1	-	-
	Feeder canal water	3	-	-
E	Pond water	6	1	1 ND
	Pond mud	6	-	-
	Soil from farm surroundings	1	-	-
	Feeder canal water	1	-	-
F	Pond water	6	-	-
	Pond mud	6	1	C
	Feeder canal water	1	-	-

ND - Not determined

Few attempts to quantify the numbers of *C. botulinum* naturally present in farm-raised fish has been made (Huss *et al.* 1974a). Huss *et al.* (1974a) detected type E in the intestinal tract of trout at the level of 5.3/g. Baker *et al.* (1990) reported 100% of the composite sample of King Salmon from Nimbus hatchery California harboured *C. botulinum* type A at a level of 23 organisms per 100g (range 4 to 120). *Clostridium*

botulinum count of 70/100g in the intestine sample of farmed fish observed in the present study was comparable to that quoted by Baker *et al.* (1990). Eklund *et al.* (1984) reported that the intestines of fish with botulism symptoms contained type E bacteria ranging from 75 to 110000 per g. In whole shrimp, the botulinum count observed in the present study was 150/100g. *Clostridium botulinum* was detected in wild shrimp in Indonesia at a level of 2/kg (Dodds 1993b).

Table 3: Distribution of *Clostridium botulinum* in farmed fish and shrimp

Farm	Sample	Type of sample tested	No. of samples tested for *C.botulinum*	No. of samples positive for *C.botulinum*	Type of toxin
A	Shrimp - *Penaeus monodon*	Whole	3	2	C,D
B	Shrimp - *P. monodon*	Whole	4	-	-
C	Shrimp - *P. monodon*	Whole	2	-	-
D	Fish - *Oreochromis mossambicus*	Skin with muscle intestine	1 1	- -	- -
E	Fish - *Chanos chanos*	Skin with Muscle Intestine	4 4	- 1	- C
	Mugil cephalus	Skin with Muscle Intestine	4 4	- -	- -
	Shrimp - *Penaeus indicus*	Whole	2	1	D
F	Fish - *Oreochromis mossambicus*	Skin with Muscle Intestine	2 2	- 1	- D
	Shrimp - *P. indicus*	Whole	5	1	D
	Shrimp - *P. indicus*	Shell with Muscle Intestine	4 4	- -	- -

Table 4: Quantitative estimation of *C. botulinum* in fish, shrimp and farm environment

Farm	Sample positive for *C. botulinum*	MPN count/100g
A	Water	4*
	Mud	15
	Mud	1100
	P. monodon	70
	P. monodon	150
E	Water	4*
	Chanos chanos	70

*Count/100ml

Table 5: Matrix of correlation of *C. botulinum* count in *P. monodon* with pond water

	MPN count of *C. botulinum*	PH	D.O.	Salinity	*P. monodon*
MPN	1.0000				
PH	0.7847	1.0000			
DO	0.3205	0.6990	1.0000		
Salinity	-0.5242	-0.6362	-0.9301*	1.0000	
P. monodon	-0.1597	0.4551	0.7000	-0.4960	1.0000

* indicates $p<0.05$

Table 6: Matrix of correlation of *C. botulinum* count in *P. monodon* with pond mud

	MPN count of *C. botulinum*	PH	D.O.	Salinity	*P. monodon*
MPN	1.0000				
PH	0.9483	1.0000			
DO	0.7204	0.7040	1.0000		
Salinity	-0.7001	0.8273	0.7095	1.0000	
P. monodon	0.9991*	0.9387@	-0.6693	-0.6693	1.0000

* - $p<0.10$; @ - $p<0.05$

Huss (1981b) reported that the prevalence and distribution of *C. botulinum* spores in fish were usually associated with their distribution in sedimentary deposits. Our findings also agree with that of Huss (1981b). Types C and D are the predominant type in tropical waters (Mortojudo *et al.* 1973; Tanasugarn 1979; Huss 1980; Haq and Suhadi 1981; Lalitha and Surendran 1992). Similar results were also observed in the present study.

Titres of *C. botulinum* toxins in enrichment cultures of fish and shrimp in Robertson's cooked Meat Medium were low (10-20 MLD/ml). Cann *et al.* (1975) reported a toxin titre of 10-4000 MLD/g in farmed trout in Great Britain. The low toxin titre in this study indicates a low contaminating load of *C. botulinum* and/or poor toxigenicity of the organisms.

The association of *C. botulinum* type A and B with neutral to alkaline soils and also with soils low in organic matter has been reported earlier (Smith 1975). A similar relationship of *C. botulinum* types C and D from sediments with pH was observed in the present study. Ortiz and Smith (1994) attributed the recent high prevalence of types C and D in U.K. to the rotting organic matter and generated heat associated with land fill sites. The high atmospheric temperature in the tropics and the rotting organic matter in the farms may favour the multiplication of types C and D in the fish/shrimp farms.

The safety of farmed fish has been questioned after an outbreak of type E botulism in Germany associated with farm-raised smoked trout fillet (Bach *et al.* 1971). *Clostridium botulinum* types C and D as the cause of human botulism is in some doubt (Smith 1990; Hauschild 1993). However, human cases of type C and D botulism have

been reported earlier (Roberts and Gibson 1979; Hauschild 1993). *Clostridium botulinum* type. D and its toxin were detected in patients who died unexpectedly with symptoms like respiratory difficulty and severe dysphagia in Switzerland (Sonnabend and Sonnabend 1981). Fastidiousness of type C in experimental monkeys (Smith 1990) and the recent infant botulism by type C in Japan (Oguma *et al.* 1990) indicated that its potential risk should not be underestimated.

The prevalence of *C. botulinum* type C and D in three farms examined indicates that if the farm conditions allow considerable enrichment of the numbers of *C. botulinum,* it will not be a danger as such, but the number of fish/shrimp marketed containing *C. botulinum* spores is likely to increase. Huss *et al.* (1974a) found that excess feed and fish faeces lying in the pond bottom provide ample nutrients for growth of *C. botulinum.* Burns and Williams (1975) have pointed out that the organically rich mud is rendered anaerobic by the deposition of food waste, dead fish etc. allowing the multiplication of *C. botulinum* and contamination of more and more fish in the farms. Eklund *et al.* (1984) have reported that fish dying from other fish diseases or even death of a few fish during transfer from one pond to another have been sufficient for the multiplication of *C. botulinum* in the farm and contamination of the fish.

Fish botulism is associated with *C. botulinum* type E (Huss and Eskildsen 1974; Cann and Taylor 1982, 1984; Eklund *et al.* 1982, 1984; Roberts 1989; Inglis *et al.* 1993). Therefore recovery of *C. botulinum* types C and D from the farms examined in the present study do not pose any great hazard to farm animals.

An understanding of the prevalence and natural spore load of *C. botulinum* in fish and their environment is essential to evaluate the potential risk of botulism from such food and the effectiveness of a processing method is affected by the initial number of C. *botulinum* spores. The incidence of *C. botulinum* was high in farmed fish/shrimp (18-20%) compared to pond mud (11%). The types C and D *C. botulinum* prevalent in water and mud were encountered in farmed fish/shrimp indicating a close link in the distribution of *C. botulinum* in fish/shellfish and sediments. The numbers of *C. botulinum* estimated from farmed fish/shrimp do not exceed those reported for wild fish from tropical waters or farmed fish from temperate countries. Only the viscera of fish were shown to be contaminated with *C. botulinum*. Therefore, efficient evisceration and washing of fish may reduce the level of contamination. The recommendations of Huss *et al.* (1974) and Cann *et al.* (1975) for the control of *C. botulinum* in trout farms apply equally well in fish farms in the present study. Adoption of safe farm management practices and following of Hazard Analysis at Critical Control Points (HACCP) can ensure production of farmed fish safe for human consumption (Gopakumar 1998). The level of contamination of pond mud by *C. botulinum* is high compared to farmed fish/shrimp. By periodical cleaning of the pond bottom, the contamination can be kept at low level. The top layer of bottom sediments of the ponds should be removed and the pond bottom mud be treated with quicklime after each farming operations to prevent any build up in the number of C. *botulinum* present in the bottom. It is also suggested that dead fish should be removed from the farms as soon as they are found, appropriate feeding strategy should be adopted to regulate excess feed to reduce the organic load of the farm and the dissolved oxygen level should be maintained at the optimum level by

aeration/exchange of water to prevent the multiplication of *C. botulinum* in the culture system.

In the present study, the numbers of bacteria isolated from farmed fish and shrimp did not exceed those reported in wild fish and the numbers of *C. botulinum* that may present a significant risk of food-borne illness is low. Therefore, farm raised fish and shrimp do not pose a great hazard to human health than do wild fish. Since aquacultured fish can be brought to market faster than wild fish, they probably pose a considerable lower microbiological risk than wild fish. The preference for 'fresh and fresh like' foods and minimally processed foods has considerably increased in recent years. The risk of botulism can be increased by such fish preservation process as they foster the growth of *C. botulinum* if processing is not proper. Therefore, the recovery of these pathogens from farmed fish and shrimp emphasizes the need for promoting good sanitation and appropriate methods of preservation to ward off post process contamination. Proper handling and quality control immediately after harvest should result in high quality and safe products from farm raised fish and shellfish.

4.1.2 Distribution of Clostridium botulinum in coastal waters of India

The distribution of *C. botulinum* in the mud samples from west coast and East coast and bivalve samples caught from the wild as well as from open farms were examined and summarised in table 7. Of the 28 mud samples analysed from the west coast, 8 samples harboured *C. botulinum* (29%) with type D predominating (5/8) followed by type A (2/8) and type C (1/8). The contamination level of West Coast mud with *C. botulinum* ranged from 0-70 per 100g. In the east coast, of the 16 mud samples

examined, *C. botulinum* was detected in 25 percent of the samples. Type C was found in the positive samples. The concentration of *C. botulinum* spores in the East Coast mud was 0-40 per 100g. The overall incidence of *C. botulinum* in mud samples was 27% with types C and D predominating followed by type A.

Table 7: Incidence of *Clostridium botulinum* in mud and bivalves from coastal waters of India

Source of sample	No. of samples tested	Sample	No. of samples positive for *C. botulinum*	MPN count of *C. botulinum* per 100g	*C. botulinum* type identified
Narakal	6	West coast mud	1	-	Type C
Kannamali	4	West coast mud	-	-	-
Andhakara-nazhi	4	West coast mud	2	40	Type A
Calicut	4	West coast mud	-	-	-
Mangalore	4	West coast mud	2	40	Type D
Veraval	6	West coast mud	3	70	Type D
Mandapam	12	East coast mud	3	40	Type C
Rameswaram	4	East coast mud	1	-	Type C
Narakal	6	Wild green mussel - *Perna viridis*	2	40	Type C
Narakal	6	Farmed oysters - *Crassostrea madrassensis*	2	-	Type D
Kannamali	3	Wild Brown Mussel - *Perna indica*	1	-	Type C
Andhakara-nazhi	8	Farmed Green Mussel - *Perna viridis*	2	70	Type A
Calicut	6	Wild green Mussel - *Perna viridis*	-	-	-

Farmed bivalve samples from the open brackishwater and marine areas of the West Coast were examined for *C. botulinum. Clostridium botulinum* was found to be present in 29 percent of the 14 samples. Types A and D were found in two samples each. In wild bivalves, the incidence of *C. botulinum* was 20% (3/5). Type C was predominant in wild shellfish samples. The estimated level of *C. botulinum* in farmed bivalves samples ranged from 0-70 per 100g. In wild samples, *C. botulinum* spore level was in the order of 0-40 per 100g. While the overall contamination level was 24% in bivalves, it ranged from 20% in wild green/brown mussel, 25% in farmed green mussel to 33% in farmed oysters.

The results of the survey of coastal areas indicate that aquatic environment is contaminated with *C. botulinum,* the counts are very low, usually less than 100/100g in sediments. In contrast, high counts of *C. botulinum* spores have been reported in sediments from Green Bay (100-36,00/g; Sugiyama *et al.* 1970), Pacific coast of the United States of America (54-3200/100g; Eklund and Poysky 1967) and Denmark (80-35000/100g; Huss, 1981b).

The occurrence of *C. botulinum* in freshwater and marine muds has been reported from many parts of the world (Huss 1981; Haq and Suhadi 1981; Dodds 1993a). Extensive surveys of sediments showed very high incidence of *C. botulinum* in the US Pacific coast and Atlantic coast of the United States (12-87%) with types A, B, C, E and F (Eklund and Poysky 1967; Craig and Pilcher 1967; Huss 1980, 1981b) and low incidence (4-8%) in the US Gulf coast, Atlantic coast (Ward *et al.* 1967 a, b) and Mobile bay (Pressnell *et al.* 1967) with types A-E. In sediments from the coast of U.K., incidence was low whereas in samples from Scandinavian waters, Norfolk Broads and

Greenland coast incidence of *C. botulinum* type E was high (86-100%; Huss 1980, 1981b; Lund 1986). In Japan, incidence of *C. botulinum* was 12% in sediments from Seto Inland sea and 28% from Ohta river and Hiroshima coast (Venkateswaran *et al.* 1989 a, b). In Iran, 17% of the sediment samples harboured *C. botulinum* (Huss 1981). Earlier survey of sediments in Indonesia demonstrated *C. botulinum* types A through E in 16% of the samples (Mortojudo *et al.* 1973). In a later survey, 12% of sediment samples were found to harbour *C. botulinum* types A-F (Haq and Suhadi, 1981) with types C and D predominating. In another survey, Suhadi *et al.* (1981) reported incidence of *C. botulinum* in 2% of the sediment samples from Indonesia with type B predominating followed by D, C and A. In Thailand, Tanasugarn (1979) reported incidence of *C. botulinum* type D and E in 2% of the sediment samples with type D predominating. In Bangladesh, Huss (1980) demonstrated *C. botulinum* types C and D in 50% of brackishwater sediments and 37% of fresh water sediments. The occurrence of *C. botulinum* type D was reported from the brackish water sediments in India (Lalitha and Surendran, 1993). *Clostridium botulinum* types C and D were detected in sediment samples from Africa (Smith 1987), United States (Ward *et al.* 1967; Pressnell *et al.* 1967) and Europe (Lund 1986). The overall incidence of 27% in mud samples in India as reported in the present study was lower than that of the United States, Europe and Bangladesh and was comparable to that of Japan. The predominance of *C. botulinum* types C and D in the tropical waters reported by Haq and Suhadi (1981), Tanasugarn (1979) and Huss (1980) has been confirmed.

In bivalve samples, an overall contamination level of 24% was found in the present study. Type C predominated in this study followed by type D and A. Pressnell

et al. (1967) found incidence of *C. botulinum* type E in 3%of oysters from Mobile bay. In contrast, Craig *et al.* (1968) found *C. botulinum* in 33% of the oyster samples, 23% of the clam samples and 68% of the crab samples from Pacific coast of the United States with type E predominating followed by types A and B. Baker *et al.* (1990) reported contamination by *C. botulinum* type A in one out of 26 shellfish samples (4%) from U.S. East coast. In Europe, Huss (1981) reported incidence of *C. botulinum* type E in 21% of the shellfish samples from the Scandinavian coastal waters and the Baltic while the contamination level is low (7%) in shellfish from Skagerrak and North sea. In Indonesia, a contamination level of 20% (6/30) was reported by Mortojudo *et al* (1973) and in a later survey Suhadi *et al.* (1981) reported contamination by *C. botulinum* types A and C at a level of 2/kg in 1% of the shellfish samples. The association of *C. botulinum* types A, C and D with shellfish was found in the present study as reported earlier by other workers (Ward *et al.* 1967a, b; Mortojudo *et al.* 1973; Suhadi *et al.* 1981). The *C. botulinum* types prevalent in the mud samples from the coastal waters were also demonstrated in bivalve samples from the same waters.

Clostridium botulinum type A is the causative agent of human botulism and type A toxin has the highest lethal activity in the genus clostridium. An outbreak of botulism associated with *C. botulinum* type A following consumption of home-processed mussels has been reported in New Zealand (Hauschild 1993). Growth and toxin production by *C. botulinum* in scallops, oyster and clam substrates were also reported earlier (Strasdine and Kelly 1967; Patel *et al.* 1978; Goldmintz *et al.* 1983; Fletcher *et al* 1988). The potential of type C botulism in humans has also been indicated earlier. Therefore, potential risk of bivalves contaminated with *C. botulinum* should not be ignored.

The recent increase in aquaculture of fish/shellfish in the coastal waters makes it important to know the ecology of *C. botulinum* in various environments. Bivalve molluscs because of their filter feeding ability, accumulate high numbers of pathogens from the environment which presents a risk to public health if toxigenic strains multiply to high numbers during improper storage and handling of shellfish (Austin and Austin, 1993). The coastal waters of India have the potential for mariculture development and it is of considerable importance to ascertain the degree of the risk of contamination with *C. botulinum* in farmed fish/shellfish from these waters. Also, chances of mishandling of shellfish during transportation as well as by retailers and consumers, are ever present. Since *C. botulinum* can cause food-borne illness in humans, anything which influences the population of *C. botulinum* in food may affect its safety.

The present study indicated that the incidence of *C. botulinum* was higher in the West coast mud (29%) compared to east coast mud (25%). The overall contamination level in bivalves was 24%. The incidence of *C. botulinum* in the aquatic environments of India is found to be lower than that of temperate water but considerably higher than that of other tropical waters. *Clostridium botulinum* types C and D predominate in the aquatic environment of India. The distribution of *C. botulinum* in shellfish is closely related to their occurrence in sediments. The level of contamination of farmed and wild shellfish is very low. Therefore it will not be a danger as such. Psychrotrophic strains of *C. botulinum* are noticeably absent in shellfish. The presence of *C. botulinum* type A in bivalves may have public health implications. In view of the prevalence of *C. botulinum* type A in bivalves the potential exists for the occurrence of cases of human botulism from consumption of bivalves which have not been processed adequately to destroy the

organism or toxin. Hence 24% recovery of these pathogens from shellfish is a matter of great concern for public health. It is of considerable importance to adequately remove these pathogens from bivalves either by self-purification in clean sea water or by adequate processing to make it fit for human consumption.

4.1.3 Investigations on the incidence of *Clostridium botulinum* in Cultivable fish/shellfish and trash fish

Twenty samples of fish belonging to eleven culturable species and twenty four samples of shellfish, belonging to six cultivable species, caught from the wild, were examined for *C. botulinum*. Of the 20 samples of fish examined, 20 percent harboured C. *botulinum* (Table 8). The frequency of contamination on the surface of fish was 20 percent while that in intestine was 15 percent. Type D predominated in surface samples (2/4) followed by type A (1/4). One sample could not be type identified as the toxin level in the enrichment was too low. Of the three positive intestine samples, only two could be type identified. Type A and C were detected in one sample each. In shellfish, twenty five percent of the samples (6/24) were found to be contaminated with *C. botulinum* (Table 9). Except clams, all the species of shellfish analysed were found to be contaminated with *C. botulinum* with type C predominating (3/6) followed by type D (2/6) and type A (1/6). The incidence of C. *botulinum* was slightly higher in shellfish than fish. Types C and D predominated in shellfish and fish. In wild freshwater fish and shellfish examined, C. *botulinum* was detected in 2 out of 10 samples (20%).

Thirty samples of trash fish collected from various sources were examined for *C. botulinum* and the results are summarized in table 10. Twenty seven percent of the

samples showed contamination by *C. botulinum*. Twenty five percent of samples showed contamination by *C. botulinum* on the surface with types A, C and D in two samples each and type B in one sample. Incidence of *C. botulinum* in the intestine samples of fish was low (5%) and type A was found in the positive sample.

In the present study, an overall contamination level of 24 percent was noticed with *C. botulinum* type C predominating followed by types D, A and B. The level of contamination at the retail level was high in the United States (Dodds 1993 b). A high level of contamination by *C. botulinum* was reported in fish from the pacific coast of the United States (5-43%; Craig and Pilcher 1967; Eklund and Poysky 1967. Craig *et al.* 1968), in fresh dressed rock salmon from California (100%; Lindorth and Genigeorgis 1986), in fresh king salmon from Alaska (100%; Garcia and Genigeorgis 1987), in fresh retail fish from California (22% and 67%; Baker *et al.* 1990) and in fish from Scandinavia (63%; Johannsen 1965 a; Huss 1981 b). Incidence of *C. botulinum* was low in the Atlantic and Gulf coast of the United States (1-5%; Nickerson *et al.* 1967; Ward *et al.* 1967 a b), in Latin American waters (2%; Ward *et al.* 1967 c), in Europe with the exception of Scandinavia (0-8%; Cann et al 1965 a; Baumgart 1972; Hobbs 1976), in Japan (4-13%; Kanzawa *et al.* 1970; Yamamoto *et al.* 1970; Haq and Sakaguchi 1980; Jarvis and Patel 1980) and in Gulf of Thailand (1%; Tanasugarn 1979) whereas in Indonesia (2-17%; Mortojudo *et al.* 1973; Haq and Suhadi 1981; Suhadi *et al.* 1981) and India (3-32%; Lalitha and Iyer 1990; Lalitha and Surendran 1992), the incidence was slightly high. Type E is the most dominant type found in aquatic environments reported in world wide studies of temperature geographical areas (Hobbs 1976; Huss and Pedersen 1979; Huss 1980; Dodds 1993 b). However, incidence of types C and D were also

reported in marine environments of the temperate areas (Carroll *et al.* 1966; Ward et al 1967 a b c; Pressnell *et al.* 1967). Baker *et al.* (1990) found predominance of type A in fish from California super market. Types C and D are the most prevalent types in the aquatic environment of South Africa (Smith 1990), Japan (12-28%; Azuma and Itoh 1987; Venkateswaran *et al.* 1989 a b), Indonesia (12%; Mortojudo *et al.* 1973; Haq and Suhadi 1981; Suhadi *et al.* 1981), Gulf of Thailand (1%; Tanasugarn 1979), Bangladesh (50%; Huss 1980) and India (Lalitha and Surendran 1992, 1993). The overall contamination level of 24% in fish as observed in the present study was low compared to the incidence in the United States and Scandinavia, but very near to that in Indonesia.

In freshwater fish and shellfish samples examined in the present study, incidence of *C. botulinum* was 20% (2/10) and all positive samples harboured types C and D. Contamination of wild freshwater fish was reported with *C. botulinum* types B and E (6-57%) in the United States (Bott et al 1966; Craig et al 1968; Chapman and Naylor 1966), with types C, E and F in Japan (Yamamoto *et al.* 1970; Azuma and Itoh 1987) and with types C, D and E (14%) in Indonesia (Haq and Suhadi 1981). *C. botulinum* was not found in wild freshwater fish in Europe (Huss 1981b).

The high incidence of *C. botulinum* in both marine and freshwater fish and shellfish samples reported in the present investigation can be attributed to the heavily contaminated environment. *Clostridium botulinum* has a wide spread distribution as reflected by its isolation from different sources. The frequency of contamination of fish was more on the surface than in the intestine. Huss and Pedersen (1979) have also reported that contamination of gut with *C. botulinum* is most common in bottom feeding fish and in pelagic fish surface is more contaminated than gut.

Table 8: Incidence of *Clostridium botulinum* in cultivable fish caught from the wild

Sl. No.	Source of the sample	No. of samples tested	Common English name of the fish	Scientific name	Sample portion examined	No. of samples positive for *C.botulinum*	*C. botulinum* Type present
1.	Cochin retail market	3	Mullet	*Mugil cephalus*	Skin with muscle Intestine	1 -	D -
2.	Cochin retail market	1	Mullet	*Liza parsia*	Skin with muscle Intestine	1 1	A *ND
3.	East coast onboard vessel	1	Reef cod	*Epinephelus tauvina*	Skin with muscle Intestine	- -	- -
4.	West coast onboard fishing vessel	3	Reef cod	*Epinephelus malabaricus*	Skin with muscle Intestine	2 2	1D,1ND 1A, IC
5.	West coast onboard fishing vessel	1	Reef cod	*Epinephelus albomarginatus*	Skin with muscle Intestine	- -	- -
6.	East coast onboard vessel	1	Red snapper	*Lutjanus argentimaculatus*	Skin with muscle Intestine	- -	- -
7.	Cochin retail market	2	Tarpon	*Megalops cyprinoides*	Skin with muscle Intestine	- -	- -
8.	Cochin retail market	2	Pearl spot	*Etroplus suratensis*	Skin with muscle Intestine	- -	- -
9.	Cochin retail market	2	Milk fish	*Chanos chanos*	Skin with muscle Intestine	- -	- -
10.	Muhamma	2	Mrigal (freshwater fish)	*Cirrhinus mrigala*	Skin with muscle Intestine	- -	- -
11.	Muhamma	2	Rohu (freshwater fish)	*Labeo rohita*	Skin with muscle Intestine	- -	- -

ND Not Determined

Table 9: Incidence of *Clostridium botulinum* in cultivable shellfish caught from the wild

Sl. No.	Source of the sample and the environment from where samples collected	No. of samples tested	Common English Name	Scientific Name	sample portion exa-mined	No. of samples positive for *C. botulinum*	*C. botu-linum* type present
1.	Fort Cochin landing centre (marine)	3	Indian white prawn	*Penaeus indicus*	Whole	1	C
2.	Cochin retail market (freshwater)	6	Fresh water prawn	*Macrobrachium rosenbergii*	Whole	2	C, D
3.	Cochin retail market (brackish water)	3	Clam	*Meretrix meretrix*	Whole	-	-
4.	Narakal (marine)	2	Green mussel	*Perna viridis*	Whole	-	-
5.	Vaikom (brackish water)	3	Green mussel	*Perna viridis*	Whole	-	-
6.	Kannamali (marine)	3	Brown mussel	*Perna indica*	Whole	1	C
7.	Andhakara-nazhi (marine)	2	Green mussel	*Perna viridis*	Whole	1	A
8.	Narakal (brackish water)	2	Oyster	*Crassostrea madrasensis*	Whole	1	D

Table 10: Distribution of *Clostridium botulinum* in trash fish from Indian waters

Sl. No.	Source of Sample	Common English name	Scientific name	Sample portion examined	No. of samples tested	No. of samples +ve for *C. botulinum*	C.botu-linum type present
1.	Cochin retail market and onboard fishing vessel	Ribbon fish	*Lepturacanthus savala*	skin with muscle	4	2	A, B
2.	Cochin retail market	Tarpon	*Megalops cyprinoides*	skin with muscle	2	-	-
3.	Cochin retail market	Pony fish	*Leiognathus splendens*	skin with muscle Intestine	4 4	- -	- -
4.	Cochin retail market	Croaker	*Johnius dussumieri*	skin with muscle Intestine	2 2	- -	- -
5.	Cochin retail market	Lizard fish	*Saurida tumbil*	skin with muscle Intestine	4 4	2 1	C A
6.	Onboard fishing vessel	Russels scad	*Decapterus russelli*	skin with muscle Intestine	2 2	1 -	D
7.	Cochin retail market	Flat fish Malabar tongue sole	*Cynoglossus macrostomus*	skin with muscle Intestine	2 2	1 -	D -
8.	Cochin retail market	Croaker Lesser tiger tooth croaker	*Otolithes cuvieri*	skin with muscle Intestine	3 3	- -	- -
9.	Cochin retail market	Big jawed jumper/ white fish	*Lactarius lactarius*	skin with muscle Intestine	3 3	1 -	A -
10.	Cochin retail market	Carangid	*Alepes djeddaba*	skin with muscle Intestine	2 2	- -	- -
11.	Cochin retail market	White sardine	*Escualosa thoracata*	Whole	2	1	C

Clostridium botulinum types C and D are the predominant types in fish and shellfish in the present study as reported for tropical areas in earlier studies (Suhadi *et al.* 1981; Haq and Suhadi 1981; Tanasugarn 1979; Huss 1980). Haq and Suhadi (1981) have attributed the dominance of types C and D along the equator to the higher temperature since 42°C is the optimum for their growth. Though types C and D have been implicated in earlier outbreaks, either type as the cause of human botulism is in some doubt (Hauschild 1993). However, the recent occurrence of infant botulism by type C in Japan (Oguma *et al.* 1990) and the fastidiousness of type C in experimental monkeys (Dolman *et al.* 1961) indicates that its potential risk should not be ignored. The recorded cases of human botulism are mainly due to types A, B and E (Smith 1990).

It is evident from the present investigation that *C. botulinum* types A, B, C and D are associated with fish species that are used in aquaculture as well as trash fish that are used as feed in the farming of carnivorous fishes. More important implication of the present finding is that the contaminated feed can be a source of C. *botulinum* spores in the aquaculture system. The contaminated trash fish feed may contaminate the farmed fish and farm environment. The contaminated trash fish feed were implicated in the botulism outbreaks in trout farms in Europe and the United States (Huss 1981 b; Eklund *et al.* 1984).

The incidence of *C. botulinum* in shellfish (25%) was higher than in fish (20%). In wild freshwater fish/shellfish, incidence of *C. botulinum* was 20%. An overall contamination level of 24% was noticed predominantly with *C. botulinum* type C followed by types D, A and B. The contamination of fish was more on the surface than the intestine. The present study indicates that the overall contamination level of

fish/shellfish (24%) does not exceed that reported for wild fish from temperate waters and is also within the range reported for tropical waters. The present study reveals that marine, brackishwater and freshwater environments in India are contaminated with *C. botulinum*. The presence of *C. botulinum* types A and B, which are associated with human botulism in fish and shellfish caught from the wild may have both ecological and public health implications. Psychrotrophic strains of *C. botulinum* are noticeably absent. Natural seed resources as well as hatchery seed resources are used in the farming of fish and shellfish. There is the possibility of transfer of live fish and fish eggs from country to country. With such transfers there is possibility of introduction of psychrotrophic *C. botulinum* types from exotic fish/fish eggs. Therefore contamination of the culture system is also possible with *C. botulinum* types associated with wild fish and shellfish through water, seed from natural sources or trash fish feed.

Clostridium botulinum types A and B could potentially grow and produce wound botulism if it is introduced into damaged tissue of humans. Moreover, the potential of type C infant botulism exists. Therefore, 27% recovery of these pathogens from trash fish is a matter of great concern while handling the contaminated fish in culture systems. Trash fish if used as feed in the culture of carnivorous fish should be properly handled and adequately processed to destroy the organism or toxin, to keep the contamination at low level and to prevent infection of farm animals and man.

4.2 *Clostridium botulinum* in processed fishery products

4.2.1 Incidence of *Clostridium* botulinum in chilled stored farmed shrimp

Results of the chilled storage study of farm-raised shrimp, *P. indicus*, are

presented in Table 11. Initial analysis of *P. indicus* showed contamination by *C. botulinum* type D at the spore level of 40 per 100 g. The toxin level in CMM enrichment cultures reached 10^3MLD/g. *Clostridium botulinum* type D was detected in shrimp samples withdrawn after 8 and 12 days of chilled storage. The *C. botulinum* count in the shrimp samples remained same after 12 days of chilled storage. Neither growth nor toxin production of *C. botulinum* was observed in shrimp during chilled storage for 12 days but toxin production was observed when samples were vacuum-packed and incubated at ambient temperature (30±1°C) for 5-8 days. The packs were bulged due to gas production and the shrimp samples were discoloured. They had also offensive odours.

Table 11: Incidence of *Clostridium botulinum* in farm-raised shrimp during chilled storage

No. of days of storage	Enrichment medium	MPN count of *C. botulinum* per 100g	Type identified	Toxin level in enrichment medium
0	CMM Whole shrimp vacuum packed	40 -	D D	$<10^3$MLD/g.
4	CMM	-	-	-
8	CMM	-	D	-
12	CMM whole shrimp vacuum packed	40 -	D D	$<10^3$MLD/g -

Recovery of spores from chilled stored shrimp in the present study indicates that spores of *C. botulinum* are highly resistant to chilling. Similar observations were made earlier by Genigeorgis and Riemann (1979) and Garren *et al.* (1994). Bartl (1973) had reported stability of *C. botulinum* toxin to chilling temperature.

The present investigation also showed the ability of *C. botulinum* to grow and produce toxin in chilled stored shrimp which were later packed under vacuum and stored at 30°C. Neither growth nor toxin production of *C. botulinum* were noticed in shrimp during chilled storage. The risk of *C. botulinum* can be increased by vacuum packing/ modified atmosphere.

Schofield (1992) reported *C. botulinum* as a potential hazard in chilled vacuum-packed foods which usually have minimal heat processing and are not heated or only mildly heated prior to consumption. Lerke and Farber (1973) studied the ability of *C. botulinum* to grow and produce toxin in shrimp meat and found growth and toxin production of types A and B during storage at 75°F (23.8°C) and of type E during storage at 50°F (10°C) in inoculated shrimp homogenates and previously heat pasteurized shrimp in air-impermeable plastic pouches. In many countries, there has been great concern recently about the safety of foods including the chilled ones. The occurrence of *C. botulinum* and their current importance in chilled foods has been discussed by Gibson (1990). The effects of Modified Atmosphere Packaging combined with chilling on the storage life of fish have been extensively studied (Cann 1984; Beirne 1990; Wignall *et al.* 1990). Since psychrotrophic *C. botulinum* strains can grow at chill temperatures, careful temperature control of chilled foods is essential both for quality and safety. Garren *et al.* (1994) investigated the potential for *C. botulinum* outgrowth and toxin production in inoculated vacuum-skin packaged shrimp and found toxin production by *C. botulinum* at 10°C after 6 days. In the present investigation, psychrotrophic *C. botulinum* strains were not detected in shrimp.

The present study reports incidence of *C. botulinum* type D in farmed shrimp during chilled storage. The spores of these strains survived chilling temperature and there may be potential for multiplication and toxin production by *C. botulinum,* if the shrimp is vacuum packed and stored without maintaining the storage temperature at or below 4°C. Although psychrotrophic *C. botulinum* strains were not isolated, it should not be assumed that these bacteria are absent in farmed shrimp. High standards of hygiene in shrimp farms and processing facilities are essential to keep the contamination at low level in order to ensure safety of aquacultured products.

4.2.2 Incidence of *Clostridium botulinum* in frozen fishery products

Fortyone samples of frozen fishery products were analysed for the presence *C. botulinum.* Six samples (15%) were found to be contaminated with *C. botulinum* types A, B, C and D (Table 12). Types C and D predominated in frozen fish/shellfish followed by type A and B. The toxin level in the positive enrichment cultures ranged from 10 to <100 MLD/ml. The frozen farmed shellfish were found to harbour *C. botulinum* at a level of 0-40 per 100 g.

Contamination of fish with *C. botulinum* is well known (Hobbs 1976; Huss 1981; Smith 1990; Dodds 1993a). Dodds (1993b) reviewed the distribution of *C. botulinum* in processed fishery products. Insalata *et al.* (1967) found a lower level of contamination (10%) with *C. botulinum* type E spores (70/kg) in commercial vacuum packed frozen flounder from Nova Scotia. Thatcher *et al.* (1967) reported incidence of *C. botulinum* in very few samples (<1%) out of 400 frozen packaged fish from Canada and types A, B and E were detected in the positive samples. Incidence of *C. botulinum* in 15% frozen fish/

shellfish found in the present study is higher than the levels reported before for frozen fish. Contamination of fresh fish from Cochin with *C. botulinum* (0-32%) has already been reported with types C and D predominating (Lalitha and Iyer 1990; Lalitha and Surendran 1992). Therefore, it is to be expected that frozen fish/shellfish may also be contaminated as well. Psychrotrophic *C. botulinum* strains were not detected in frozen fishery products.

In the present study, *C. botulinum* spores were recovered from farmed shellfish which were initially shown to be contaminated with *C. botulinum* after frozen storage of 2 months. Earlier work of Georgala and Hurst (1963) and Genigeorgis and Riemann (1979) have shown that *C. botulinum* spores are highly resistant to freezing and the toxin is unaffected by freezing. Similar results were noticed in the present study. The non-proteolytic *C. botulinum* strains are capable of growth and toxin production at chilled temperatures (Hatheway 1993; Eklund 1993). Abusive storage temperature, therefore, induce toxin production by *C. botulinum* in such foods if the raw food is contaminated with *C. botulinum*.

Nearly all of the fish-borne botulism outbreaks were linked to smoked, salted, canned or fermented products, usually eaten without further cooking (CDC 1979). Frozen fishery products have never been implicated in any outbreaks of botulism. *Clostridium botulinum* types A, B and E are the causative agents of human botulism. Though types C and D are generally considered as not hazardous to humans, the potential of type C and D botulism exists. The recovery of *C. botulinum* types A, B, C and D from 15% of frozen fish is a matter of concern.

Table 12: Distribution of *Clostridium botulinum* in frozen fish and shell fish

Source of sample	Sample portion exa-mined	Sample	Total No. of samples exa-mined	No. of samples positive for *C.botulinum*	MPN count of *C. botulinum* per 100 gram	Type identi - fied
Farmed	Whole	*Penaeus indicus*	4	1	40	D
Farmed	Whole	*Perna viridis*	4	1	40	A
Wild	Whole	*Perna viridis*	4	-	-	-
Wild	Whole	*Saurida tumbil*	3	-	-	-
Wild	Whole	*Decapterus russelli*	4	1	-	D
Wild	Whole	*Sepia pharaonis*	3	-	-	-
Wild	Whole	*Pampus argenteus*	3	-	-	-
Wild	SM	*Sardinella longiceps*	4	1	-	C
Wild	SM	*Nemipterus japonicus*	4	-	-	-
Wild	SM	*Scomberomorus commerson*	4	1	-	C
Wild	SM	*Lepturacanthus savala*	4	1	-	B

Another implication of the present finding is that *C. botulinum* types associated with frozen fish may be introduced into fish farms through trash fish feed. In the aquaculture production of carrivorous fishes like Groupers, Seabass and Snapper, trash fish is used as feed. Huss *et al.* (1974 a) had reported fish botulism outbreaks associated with contaminated trash fish feed. The abusive storage temperature of the trash fish may induce toxin production by *C. botulinum* in such foods and pose a hazard to farm animals.

Incidence of *C. botulinum* in frozen fishery products was 15%. Frozen farm raised shrimp and mussel showed contamination by *C. botulinum* at the level of 40 per 100g. Psychrotrophic *C. botulinum* strains were not detected in frozen fishery products. Types C and D predominated in frozen fish/shellfish followed by type A and B.

Clostridium botulinum spores are highly resistant to freezing. There is a possibility of introduction of *C. botulinum* types A and B into fish farms through contaminated trash fish feed.

Botulism has not been reported in India so far. *Clostridium botulinum* is considered as an environmental contaminant and is found in water, soil and fish. Though frozen foods have an enviable record for bacteriological safety, it is of great importance to give constant attention to factors controlling the possible survival and transmission of *C. botulinum* in such foods. For prolonged frozen storage of fish/shellfish, strict temperature control is necessary to prevent multiplication of *C. botulinum* and to ensure quality and safety of the product.

4.2.3 Distribution of *Clostridium botulinum* in cured fishery products

A total of 40 cured fish/shellfish samples procured from local retail markets were examined for the presence of *C. botulinum* and the data are summarised in table 13 and 14. *Clostridium botulinum* was detected in 13% of the samples with type D predominating (4/5) followed by type C (1/5).

The water activity (a_w) of samples varied greatly (fig. 8). Eighty eight percent of the samples had a_w values in the range of 0.71-0.80 while 10% had a_w levels of 0.81 to 0.88. The analysis of the water content of the cured fish samples revealed that 55% (22/40) samples had water content in the range of 46-51% and in 25% of the samples, water content varied between 36-45%. In cured prawn samples, water content was still lower (<22%). The sodium chloride content of the cured fish samples varied between 16-25% in only 60 percent of the samples. 20% had sodium chloride content in the range of 11-15%. The sodium chloride content of less than 7% was noticed in 8 prawn

Hauschild (1989) reviewed the prevalence of *C. botulinum* in fresh processed fish. Salted fish from the Caspian sea were 29 percent positive while smoked fish carried spores in 1.1-20 percent of samples. Dodds (1993b) reported incidence of *C. botulinum* in 63% of salted carp from Caspian sea and type E spores were found at the level of <60/10g in salted fish from Caspian sea and 490/10g in salted carp. Incidence of *C. botulinum* type D in dried fish was reported from India (Lalitha and Surendran 1993). A low incidence of *C. botulinum* (13%) was found in cured fish in the present study.

The viability of *C. botulinum* spores at water activity level 0.75 was shown in the present study by the toxic enrichment cultures of cured fish with water activity 0.75. The viability of *C. botulinum* spores for long periods in many foods of low water activity have been reported earlier (Troller and Christian 1978; Genigeorgis and Riemann 1979; ICMSF 1980). These spores are of great concern after rehydration of such foods (ICMSF 1980). There have been outbreaks of botulism associated with salt dried fish (Troller and Christian 1978; Sakaguchi 1979; Huss 1981b; McClure *et al.* 1994).

It is noted that 56% of the cured fish samples had moisture content in the range of 46-51% and 44% had moisture content in the range of 37-45%, values higher than the Bureau of Indian Standards (BIS) (IS: 2882, 1964; IS: 5198, 1969 and IS: 2883, 1976) which prescribe a range of 10-35% moisture normally and 40-45% moisture in respect of certain big fishes (Gopakumar and Devadasan 1983). Most of the samples do not conform to the BIS levels of salt (IS: 594, 1962). It is evident from the results of the present study that majority of the cured fish at the retail level contained inadequate salt levels and high moisture levels as reported earlier (George Joseph *et al.* 1986, 1988; Kalaimani *et al.* 1988; Prasad *et al.* 1994). Kalaimani *et al.* (1988) reported a_w levels of less than 0.80 in

30 percent of the cured fish examined from Quilon, Vizhinjam and Tuticorin. In the present study nearly 90% of the samples had a_w values in the range of 0.71 to 0.80. Wide variations were also observed in the a_w levels of cured fish examined in the present study.

A survey of the cured fish sold at the retail markets in Cochin revealed wide variation in the salt concentration indicating inadequate control of the brining process in a number of products. Recently outbreaks of botulism from *Kapchunka* (salt-cured, air dried uneviscerated white fish) in USA and Israel (CDC 1987; Slater *et al.* 1989; Telzak *et al.* 1990) and from Faseikh (uneviscerated fish) in Egypt (Weber *et al.* 1993) have been reported which were attributed to poorly controlled salting process. *Clostridium botulinum* types B and E were involved in the above outbreaks.

The predominance of *C. botulinum* types C and D in fresh fish has been reported from tropical areas (Tanasugarn 1979; Haq and Suhadi 1981; Lalitha and Surendran 1992). *C. botulinum* types C and D are the predominant types in sediment samples from Bangladesh, Indonesia, Thailand and India (Huss 1980; Haq and Suhadi 1981; Tanasugarn 1979; Lalitha and Surendran 1993) and soil samples from Indonesia and Thailand (Tanasugarn 1979; Hayashi *et al.* 1981). The finding that types C and D are the predominant types in cured fish as shown by the present study is as expected.

Clostridium botulinum types C and D have been reported to cause intoxications only in animals (Smith 1990; Hauschild 1993). However, cases of human botulism due to types C and D have occurred earlier (Roberts and Gibson 1979; Hauschild 1993). Sonnabend and Sonnabend (1981) detected *C. botulinum* type D and toxin in patients who died unexpectedly with severe respiratory difficulty and dysphagia. Fastidiousness of type C in experimental and captive monkeys (Dolman *et al.* 1961; Barns and Mead

1986) and recent infant botulism by type C in Japan (Oguma *et al.* 1990) indicate a potential risk. The possible hazard due to salted fish is highlighted by the incidents of botulism associated with it.

The present study indicates a low incidence of *C. botulinum* (13%) and predominance of type C and D in cured fishery products. *Clostridium botulinum* spores remain viable at water activity (a_w) level 0.75 and are able to grow and produce toxin after rehydration of such foods. The detection of *C. botulinum* in cured fish indicate either post processing contamination or the ability of *C. botulinum* strains to survive the salting process during the manufacture of cured fish. Wide variations in the salt concentration of cured fishery products indicate inadequate control of the brining process. Therefore, the incidence of *C. botulinum* in 13 percent of the cured fishery products should emphasize the need for adequate brining and drying to protect these products until their final use. Proper hygienic conditions in the curing yards are to be encouraged for the production of good quality products.

4.3 Isolation and characterization of *Clostridium botulinum*

After incubation for three days at 30°±1°C, colonies producing pearly layer surrounding and covering the colony on TSGY agar were presumptively identified as *C. botulinum* and they were isolated and inoculated into CMM. The aerobic growth of the above isolates was checked by plating on to TSGYA followed by aerobic incubation. The isolates were type identified by toxicity test and toxin-neutralisation tests. Type cultures of *C. botulinum* types E (NCIB 10660) and D (ATCC 27517) were also purified

and type identified by toxin neutralization test. Trypsin activation was necessary only for type E strain.

Preheating treatment favoured the isolation of *C. botulinum* types A, B, C and D. Preheating at 80°C for 10 min facilitated the isolation of *C. botulinum* types C and D strains.

The morphological and biochemical characteristics of the *C. botulinum* isolates were examined using the identification scheme of MacFaddin (1980) and the results were summarised in table 15. Morphologically, the vegetative cells of all the isolates and reference strains were gram-positive rods but became gram-negative as the culture aged. Spores were produced abundantly in CMM. The spores were oval shaped and were formed subterminally causing a moderate swelling of the bacilli. Young cultures of all the isolates and reference strains showed vigorous motility.

Cultural characteristics of the *C. botulinum* isolates and the reference strains were generally similar to those reported by Mac Faddin (1980) and Sneath (1986). The cultural characteristics of proteolytic *C. botulinum* types A and B isolated and non-proteolytic C. *botulinum* type E showed some differentiating features. Type E reference strain showed negative esculin hydrolysis and hydrogen sulphide production. The most striking difference between the two cultures was their ability to attack protein. *Clostridium botulinum* types A and B isolates attacked cooked meat particles and gelatin. The main difference in saccharolytic activity between these two types was the ability of the non-proteolytic type E strain and the inability of the proteolytic types A and B to produce acid and gas from sucrose. Of the 12 strains of *C. botulinum* types A and B tested all showed characteristics typical of types A and B *C. botulinum*.

Isolates of *C. botulinum* types C and D were different from proteolytic types A and B because of acid production from galactose, melibiose, fructose, maltose and mannose and very slow digestion of meat particles in CMM and lecithinase production in egg yolk agar. Some of the types C and D isolates lost their toxigenic properties during transfer in laboratory media. All type C isolates (12/12) showed positive reactions for galactose, melibiose, fructose, maltose and mannose. But type D type culture strain (ATCC 27517) produced only a weak reaction for melibiose. A total of 15 type D cultures were isolated. The characteristics of type D isolates from sediments and farmed fish (D 1530) were generally similar to type culture strain. They were different from type culture strain (ATCC 27517) by virtue of weak galactose fermentation. Type D strain isolated from cured fish was different from type culture strain (ATCC 27517) because of weak fructose fermentation. All the types C and D strains examined gave positive esculin hydrolysis similar to the proteolytic types A and B isolates.

Biochemical characteristics such as proteolysis, sugar fermentation, lecithinase and hydrogen sulphide production of *C. botulinum* types, A, B, C and D varied with the cultures. Sneath (1986) and Willis (1990) grouped proteolytic *C. botulinum* types A and B in Group I and *C. botulinum* types C and D in group III and *C. botulinum* type E and non-proteolytic B and F in Group II based on the cultural characteristics.

Eklund and Poysky (1981) have reported loss of toxigenic characteristics in pure cultures of *C. botulinum* during culture in laboratory and they suggested that production of toxins by *C. botulinum* types C and D is mediated by bacteriophages. Similar loss of toxigenicity was observed in pure cultures of *C. botulinum* types C and D during culture in laboratory media in the present study.

Table 15: Morphological and biochemical characteristics of *Clostridium botulinum* strains isolated from fish and sediment

Characteristics	Type A strain	Type B strain	Type C strain	Type D strains			Type E strain
	53 A	90 B	18 C	2131 D	2693 D	D 25717	E 10660
Motility	+	+	+	+	+	+	+
Spore	St*	St	St	St	St	St	St
Lecithinase produced	-	-	+	-	+	+	+
Lipase produced	+	+	+	+	+	+	+
Esculin hydrolysed	+	+	+	+	+	+	-
Nitrate reduced	-	-	-	-	-	-	-
H_2S produced	+	+	(+)@	+	+	+	+-
Toxicity	+	+	+	+	+	+	+
Gelatin hydrolysed	+	+	+	+	+	+	+
Acid production from: Glucose	+	+	+	+	+	+	+
Fructose	-	(+)	+	(+)	+	+	+
Galactose	-	-	+	+	(+)	+	(+)
Lactose	-	-	-	-	-	-	-
Maltose	(+)	+	+	+	+	+	+
Mannose	-	-	+	+	+	+	+
Melibiose	-	-	+	(+)	(+)	(+)	-
Raffinose	-	-	-	-	-	-	-
Salicin	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	+
Milk reaction	d	D	dc	dc	dc	dc	c
Meat digested	+	+	+ slow	+ slow	+ slow	+ slow	-

* - sub terminal
@ - weak
d - digestion
dc - digestion and clot
c - clot

Segner *et al.* (1971 a) reported weak and delayed fermentation of maltose and melibiose by marine type C strains. Oguma *et al.* (1986) found variations in the biochemical properties of types C and D strains of *C. botulinum*. They concluded that poor cell growth may lead to false negative results. In the same study, using the marine type C strains of Segner *et al.* (1971) they demonstrated fermentation of maltose, melibiose, mannose, galactose and fructose and they attributed this discrepancy to a difference in procedure.

All the strains of *C. botulinum* type C isolated in the present study showed characteristics typical of marine type C strains of Oguma *et al.* (1986). But type D isolates showed wide variations in the biochemical properties.

The present study indicated that morphologically all *C. botulinum* types were similar. Culturally non-proteolytic type E, types C and D strains proteolytic types A and B differed considerably. *Clostridium botulinum* types A and B have been implicated in human botulism outbreaks and therefore the isolation of these types from wild and farmed fish and shell fish in the retail trade is of great significance from the point of view of public health hazards. The mere occurrence of *C. botulinum* on a food product is not a hazard. But what is more important is that viable *C. botulinum* spores present are given the opportunity to germinate and produce toxin. The isolation of *C. botulinum* types A and B from fish should certainly emphasise the need for adequate processing to prevent risk to humans by consumption of such contaminated fishery products.

4.4 Sensitivity of fish to *Clostridium botulinum* toxin

The results of the sensitivity studies of fish to *C. botulinum* toxins A to E are summarised in table 16. All five toxin types A to E were toxic to *Oreochromis*

mossambicus.

Shortly before the development of botulism signs, the fish became hypersensitive. The first signs of the disease in fish were loss of colour and hyperventillation of the gills followed by sluggish, random swimming. Due to muscular paralysis and loss of equilibrium, the fish floated on the surface, swimming on their backs and swimming head on into the sides of the fish tanks. When disturbed, they swam towards deeper water, again losing their equilibrium and occasionally surfacing on their backs, sinking and then rising to the surface to resume swimming. Because of the paralysis of the fin muscles, they were unable to maintain a horizontal balance and would sink to the bottom tail first. This behaviour was repeated until the fish died. After death, the gill covers of the fish were seen extended.

Fish was more sensitive to type E toxin. The minimum Intraperitoneal (IP) lethal dose of toxin from pure cultures of type E for fish was one half of the minimum lethal dose for mice. For toxin types A, B, C and D, *O. mossambicus* were considerably more resistant than mice. The minimum IP lethal dose of toxin from pure cultures of types A and B for fish was equal to 100 minimum lethal dose (MLD) for mice whereas that for types C and D was equal to 10 MLD for mice.

Skulberg and Grande (1967) reported that type E toxin had the same toxicity to *Salmo gairdneri* as it has for mice. In contrast, Eklund *et al.* (1984) found that the minimum IP lethal dose of toxin from pure cultures of type E for fish was one half of the minimum lethal dose for mice when the fish were in the temperature range 1 to 20°C. Similar results were noticed in the present study. Hiroki (1970) also reported sensitivity of freshwater fishes to *C. botulinum* type E toxin. Haagsma (1975) demonstrated

sensitivity of eels and carp to types C and E botulinum toxin. Cann and Taylor (1982) reported sensitivity of rainbow trout to type E toxin on intraperitoneal inoculation.

Table 16: Sensitivity of fish to *Clostridium botulinum* toxins

Type of *C. botulinum* toxin inoculated	No. of fish inoculated	Mouse lethal doses inoculated (I. P. route)	No. of fish sensitive to toxin	Hours prior to botulism symptoms in fish
Type A (53A)	3	1000	3	24-48 h
	3	100	2	48-96 h
	3	10	-	-
Type B (90B)	3	1000	3	24-48 h
	3	100	2	48-96 h
	3	10	-	-
Type C (18C)	3	100	2	24-48 h
	3	10	1	48-96 h
Type D (2693D)	3	100	3	24 h
	3	10	2	48-72 h
Type E (NCIB 10660)	3	1000	3	<18h
	3	100	3	<18h
	3	10	3	<18h
	3	1	3	24-36h

Skulberg and Grande (1967) found that *S. gairdneri* were considerably more resistant than mice for toxin types A, B, C and D. In contrast, Crisley (1960) suggested gold fish as an alternative to mice as laboratory animals for toxicity studies of *C. botulinum* type A. The observations made in the present investigation agree with that of Skulberg and Grande (1967). Skulberg and Grande (1967) reported that although trout are carriers of *C. botulinum* as a commensal in their intestines and on their gills, they are similar to man and many other animals in being susceptible to its performed toxin when it is administered either parenterally or orally.

The present study revealed that C. botulinum toxin types A to E were toxic to fish. For toxin types A to D, fish were considerably more resistant than mice and for type E toxin, fish was more sensitive. The minimum IP dose of type E toxin for fish was one half of the minimum lethal dose for mice. The present study suggests that fish can be a good alternative to mice as laboratory animals for toxicity studies of only *C. botulinum* type E. The study also indicates that under favourable environment, *C. botulinum* types prevalent in fish farms can multiply and cause infections in the farmed species. Therefore, it is suggested that good hygiene should be maintained in fish/shrimp farms to keep the contamination at low level.

4.5 Effect of temperature on the stability of *Clostridium botulinum* toxins

The botulinum toxins from *C. botulinum* types A, B, C, D and E were diluted in gelatin-phosphate buffer (pH6.2) and stored at -15°C for a period of 180 d and examined after 90 d and 180 d for the inactivation of toxin by freezing. The original toxicity was maintained throughout the storage period (table 17). Types A, B, C, D and E toxins were not inactivated by freezing.

For heat inactivation study, toxins from *C. botulinum* types A and D were used. The original toxicities of types A and D toxins in the culture supernatant of TYG medium were 2000 MLD/ml. When heated at 40°C for a period of 7 d reduced 99% of toxicity of type A toxin (table 18). Heating at 50°C over a period upto 30 min reduced 90% of the toxicity of type A toxin whereas heating at 60°C for a period of 10 min reduced the toxicity to 0.1% (table 19). The rate of thermal inactivation of type A toxin was non-linear (fig. 10). Toxicity was completely lost at pH 7.0 by heating at 60°C for 30 min, at

70°C for 10 min, at 80°C for 1 min and at 100°C for 1 min.

Standing at room temperature for 30 d at pH 6.7 resulted in gradual loss of toxicity of type D toxin (table 18). At 37°C, 80% of toxicity was lost after 7 d and incubation of toxin at 40°C for 2 d reduced toxicity to <1%. Heating type D toxin at 50°C for 30 min at pH 6.7 reduced the toxicity to <1% (Table 20) when heated at 60°C over a period upto 5 min, reduced 98.7% of the toxicity of type D toxin. The rate of thermal inactivation of type D toxin also was non-linear (fig. 10). After an initial rapid inactivation, <1% of the remaining toxin appeared to be more slowly inactivated. Heating at 60°C for 10 min inactivated only 99.3% of type D toxin and after 30 min only 99.7% toxicity was lost. But heating at 70° for 5 and 10 min and at 80° for 10 min completely destroyed the type D toxin.

The pH of most fishery products fall in the range 5.0-6.8. Therefore, the thermal behaviour of this most heat stable toxins was studied in fish muscle medium (pH 6.7) and TYG medium (pH 7.0). When heated at 60°C for 30 min toxicity of type A botulinum toxin was completely lost at pH 7.0 whereas in fish muscle medium at pH 6.7 and at the same original level of toxin, the toxicity was reduced only to 2 MLD/ml after the same treatment.

Table 17: Stability of *Clostridium botulinum* toxins during freezing

C. botulinum Toxin type	Initial concentration of toxin MLD/ml	Storage time (d)	Reduction in toxicity (%)
53 A	1.0×10^4	180	Nil
90 B	2.0×10^4	180	Nil
18 C	3.0×10^2	180	Nil
2693 D	2.0×10^3	180	Nil
E 10660	1.0×10^3	180	Nil

Table 18: Stability of *Clostridium botulinum* toxin to storage at temperatures 30°C, 37°C, 40°C

C. botulinum Toxin type	Initial mouse MLD/ml	Temperature (°C)	Time D	pH	Reduction in toxicity (%)
53 A	$2.0x10^3$	40	7	7.0	99.0
2693 D	$2.0x10^3$	30	30	6.7	80.0
		37	7	6.7	80.0
		40	2	6.7	99.4

Table 19: Thermal destruction of *Clostridium botulinum* type A (53A) toxin

Temperature °C	Time and % of reduction in toxicity (initial concentration $2x10^3$ MLD/ml)			
	TYG medium (pH 7.0)		Fish muscle medium (pH 6.7)	
	Time (min)	Reduction in toxicity (%)	Time (min)	Reduction %
50	30	90	-	-
60	1	80	-	-
	5	99	-	-
	10	99.9	-	-
	-	-	15	99.7
	30	Total	30	99.9
	-	-	1	99.9
70	-	-	5	total
	10	Total	10	total
80	1	Total	1	total
	10	Total	10	total
100	1	Total	1	total

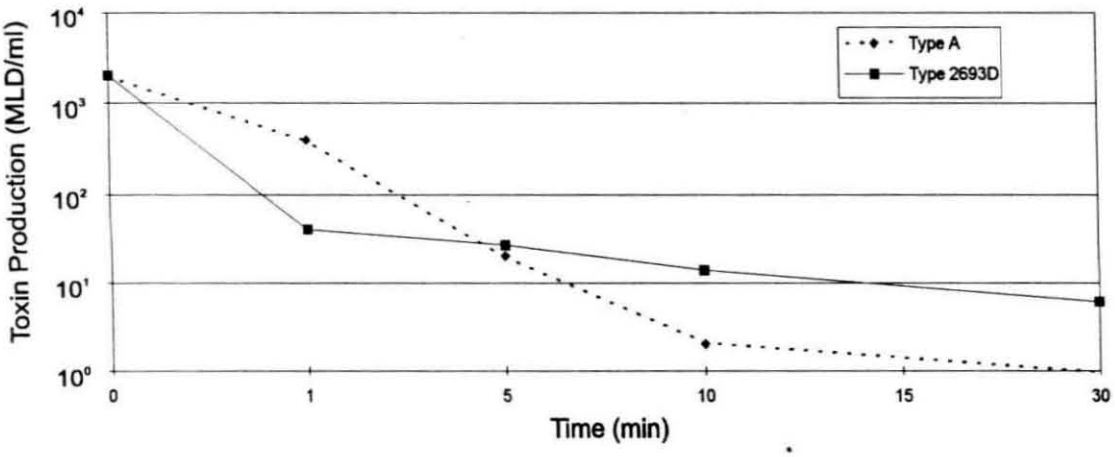

Fig. 10. Thermal inactivation of *Clostridium botulinum* type A (strain 53 A) and type D(strain 2693D) toxins at 60°C

Table 20: Thermal destruction of *Clostridium botulinum* type D (2693 D) toxin

Temperature °C	Time and % of reduction in toxicity (initial concentration $2x10^3$ MLD/ml)			
	TYG medium (pH 6.7)		Fish muscle medium (pH 6.4)	
	Time (min)	Reduction %	Time (min)	Reduction %
50	30	99.4	-	-
60	1	98.0	-	-
	5	98.7	-	-
	10	99.3	-	-
	-	-	15	total
	30	99.7	30	total
70	-	-	1	99.0
	5	Total	5	total
	10	Total	10	total
80	1	Total	1	total
	10	Total	10	total
100	1	Total	1	total

Stability of botulinum toxins to storage at -15°C (Yao *et al.* 1973), at -20°C (Woolford *et al.* 1978; Hubalek and Halouzka 1988) and at -70°C (Hubalek and Halouzka 1988) have already been reported and reviewed by several investigators (Georgala and Hurst 1963; Genigeorgis and Riemann 1979; ICMSF 1980; Huss 1981b). It has been demonstrated that *C. botulinum* type A, B, C, D and E toxins are stable at -15°C. The results of the present study confirm and extend the earlier finding that botulinum toxin is not inactivated by freezing.

Heating is the only practical means of inactivating botulinum toxins in foods and boiling of foods has been the rule. Heat inactivation studies of botulinum toxins of types A and D have demonstrated that after an initial rapid inactivation, <1% of the remaining toxin appears to be more slowly inactivated than the major fraction and hence the rate of inactivation was non-linear. Similar observations were made earlier on the heat

inactivation rates of type A, B and E (Cartwright and Lauffer 1958; Licciardello *et al.* 1967 a, b; Yao *et al.* 1973; Woolford *et al.* 1978; Bradshaw *et al.* 1979). Cartwright and Lauffer (1958) interpreted their findings as the result of a dissociation of toxins into two entities with different thermal inactivation rates. However, other explanations have also come forward, such as aggregation of toxin molecules or of denatured molecules with toxin or changes in molecular shape (Woodburn *et al.* 1979). Cartwright and Lauffer (1958) working with type A toxin in buffers found no inactivation at 40°C after 30 min, a 2 log inactivation at 50°C and a 3 log inactivation at 60°C. Heating at 60°C for 30 min reduced 99.9% toxicity at pH 6.9. Prévot and Brygoo (1953) and Woolford *et al* (1978) have reported 99.9% reduction in toxicity of type A toxin when heated at 60°C for 2 min and 68°C for 15 min respectively. Standing at room temperature at pH 6.2 or 6.8 results in a gradual loss of toxicity (Lewis 1981). The results of the present study is in good agreement with that of Cartwright and Lauffer (1958) and Lewis (1981). However, the minor variations in the thermal destruction of type A toxin noticed in the present study may be attributed to the variation in the pH value of the substrate and variations in the initial concentration of toxins.

Yao *et al.* (1973) reported that maximum heat stability of type E toxin was found with fresh toxin and on frozen storage for more than a week type E toxin lost its heat stability. Contrary to this, Woolford *et al.* (1978) have shown that the heat inactivation rates for type A remained same after frozen storage for a period of 180 d. The effect of pH and toxin titre on the heat stability of botulinum toxin were studied by several investigators (Scott and Stewart 1953; Abrahamsson *et al.* 1966; Licciardello *et al.* 1967b; Losikoff 1978; Woolfood *et al.* 1978). Studying the heat inactivation of types A

toxin in convenience foods with various pH values, Woolford *et al.* (1978) have shown that pH is the most important factor in determining heat stability of toxin. Protective effect of food constituents on the stability of botulinum toxin have also been reported (Scott 1950; Scott and Stewart 1953; Abrahamsson *et al.* 1966; Licciardello *et al.* 1967 b; Woolford *et al.* 1978, Bradshaw *et al.* 1979). Woodburn *et al.* (1979) suggested that proteins and possibly other colloidal components in foods may be a factor which lend protection to the toxins. The differences in the inactivation times of type A toxin in the present study in TYG medium and fish muscle medium when heated at 60°C for 30 min may be attributed to the protective effect of fish muscle medium.

Heating at 60°C for 30 min reduced toxicity of type D toxin in TYG medium (pH 6.7) only to <1% whereas toxicity of type A toxin was completely lost. The initial concentration of both toxins were same. In fish muscle medium toxicity of type D toxin was reduced to 1% when heated at 70°C for 1 min whereas toxicity of type A was reduced to 0.1%. The data indicates that thermal resistance of type D toxin is slightly higher than that of type A. However, heating at 80°C for 1 min inactivated both toxins completely. Similar observations were reported earlier.

Prévot and Brygoo (1953) have shown that thermal resistance of *C. botulinum* types C and D toxin is higher than that of types A, B or E. They found that a temperature of 90°C for 2 min must be reached to destroy type C and D toxin. Hubalek and Halouzka (1988) studying the thermal sensitivity of *C. botulinum* type C toxin, have shown that for 99% reduction of toxicity time required was 6 months at +5°C, 3 weeks at +20°C, 2 weeks at+28°C, 2 d at +37°C, 9 h at +42°C, less than 30 min at +56°C, less than 20 min at +60°C and below 5 min at +80°C. Woodburn *et al.* (1979) have recommended heating

85°C for 5 min as the minimum heat treatment for inactivation of botulinum toxin. The results of the present study on type D toxin is in good agreement with that of Hubalek and Halouzka (1988) but contrary to the observations of Prèvot and Brygoo (1953). The differences in these results may be attributed to the differences in the initial toxin concentration i.e. $20x10^3$ MLD/ml Versus $8.0x10^4$-$1x10^5$ MLD/ml. As Scott and Stewart (1950) have pointed out, toxins at high titre require more heat for inactivation than toxins at a low titre.

The present study revealed that freezing or subsequent frozen storage and thawing caused no destruction of type A, B, C, D and E toxins. Of greatest concern is the stability of preformed toxin in fish products which are consumed without prior heating. The biochemical changes in some fish products and animal feed do not influence toxicity during freezing or frozen storage. To reduce the botulism hazard, it is very important to keep the fish raw material chilled to near zero at all times thereby preventing formation of toxin in the final product. Standing at room temperature at pH 7.0 resulted in a gradual loss of toxicity of type D toxin. Prolonged incubation of *C. botulinum* cultures at 37°C or 40°C also resulted in a gradual loss of toxicity. Heating at 80°C for 10 min inactivated the toxin of types A and D to no detectable toxin. The results of the present study also indicates that measures to control type A toxin should also control type D toxin.

4.6 Growth characteristics of *Clostridium botulinum*

4.6.1 Influence of temperature

4.6.1.1 Influence of temperature on growth and toxin production by *Clostridium botulinum*

The influence of temperature on growth and toxin production by

C. botulinum type A to D isolated from fish and sediments and by reference strains were studied and the data are summarised in table 21. Growth and toxin production occurred at 30°C within 24h in CMM tubes inoculated with *C. botulinum* type A and B isolates from fish and reference strains of type A (NCIB 10640), type B (NCIB 10657) and type E (NCIB 10660). With the exception of one, all type C and D isolates from fish and sediment and type D reference strain (ATCC 27517) developed toxin in CMM tubes after 24h at 30°C.

The growth and toxin production by *C. botulinum* type A and B isolates were delayed for a period of 7-8 d and that of reference strains were delayed for a period of 6-7 d at 15°C. Type E reference strain (NCIB 10660) grew and produced toxin within 3 d at 15°C. Growth and toxin production by type C and D strains were delayed for a period of 7-9 d at 15°C. The outgrowth times were substantially shorter at 30°C.

At 10°C only type E could grew and produced toxin. The growth and toxin production was further delayed for a period of 8 d. Neither growth nor toxin production could be noticed at 10°C and below by *C. botulinum* types A to D isolates and the reference strains. At 4°C growth and toxin production were noticed in type E reference strain inoculated tubes after 35 d. A delay in outgrowth time occurred with the lowering of incubation temperature.

The limiting temperature for growth and toxin production by *C. botulinum* type A and B isolates was 15°C and the delay in growth for a period of 6-8 d observed in this study were reported earlier. Smelt and Haas (1978) studied the behaviour of proteolytic *C. botulinum* type A and B near the lower temperature limits of growth and found that growth and toxin production occurred within a week at 15°C. Some of the strains could

grow in 3-4 weeks at 12°C but none could grow at 10°C. Lynt *et al.* (1982) has reviewed the growth at low temperatures of proteolytic and non-proteolytic strains of *C. botulinum*. Solomon *et al.* (1982) studied the ability of proteolytic *C. botulinum* type B and F to grow at low temperatures in broth and crab meat. They have reported that type B and F grew and produced toxin in broth at 26°C and 12°C but not at 8°C and 4°C. However, growth was observed at 12°C after 100 d. Jensen *et al.* (1987) noticed negligible growth of type A strains at 12°C after 28 d of incubation. No growth occurred for strain A 69 at 8°C and 4°C. Sperber (1982), Gaze (1992), Eklund (1993) and Lund and Peck (1994) have reviewed the influence of temperature on the growth of *C. botulinum*.

Table 21: Influence of temperature on growth and toxin production by *Clostridium botulinum*

C. botulinum strains	No. of spores Inoculated	Growth and toxin production (d) (range in parenthesis)			
		30°C	15°C	10°C	4°C
Type A – 53 A	1.1×10^5	1	6 (6-7)	-	-
- NCIB 10640	2.7×10^6	1	6 (5-6)	-	-
Type B – 9 B	3.2×10^6	1	8 (7-8)	-	-
- NCIB 10657	1.5×10^5	1	6 (6-8)	-	-
Type C – 18 C	3.0×10^6	2	8 (7-9)	-	-
- 275 V	6.0×10^5	2	8 (8-9)	-	-
Type D – ATCC 27517	1.0×10^6	2	8 (8-9)	-	-
- 2693 D	5.0×10^5	2	7 (7-9)	-	-
- 2131 V	3.0×10^7	1	8 (8-9)	-	-
Type E – NCIB 10660	1.0×10^3	1	3 (3-5)	8 (8-10)	35 (34-38)

The limiting temperature for growth and toxin production by type E reference strain was 4°C in the present study. It was reported earlier that type E could grow and produce toxin at 3.3°C, 4°C and 5°C (Ohye and Scott 1957; Schmidt *et al.* 1961; Roberts

and Hobbs 1968; Solomon *et al.* 1977; ICMSF 1980). Lynt *et al.* (1982) found that type E grew within 52 d at 4°C in broth.

The limiting temperature for *C. botulinum* type C and D isolates and the type D reference strain was found to be 15°C. Segner *et al.* (1971 b) studied the minimal growth temperature of marine strains of *C. botulinum* type C and found that 15.6°C was the limiting temperature for growth in FEM medium and in ground haddock. They have indicated that the minimal temperature for growth of *C. botulinum* type C is very near to that accepted as limiting for growth of type A and proteolytic type B as observed in this study. The present investigation revealed that type D isolates and the reference strain behaved similar to type C isolates. Informations on the growth characteristics of type D are scanty.

The lag phase for type A and B isolates increased with the lowering of storage temperature from < 24h at 30°C to almost 7 d at 15°C. The effect of temperature on lag phase duration and rate of growth of *C. botulinum* type A, B and E were evaluated by Ohye and Scott (1953, 1957). They have observed a lag time of 8 h at 30°C and 7 d at 15°C. Jensen *et al* (1987) observed growth of *C. botulinum* type A strains after 5 d at 16°C. The results of the present study agreed well with that of Ohye and Scott (1953, 1957) and Jensen *et al* (1987).

The lag phase for type E reference strain increased with the lowering of storage temperature from <1 d at 30°C to 3 d at 15°C and to more than 8 d at 10°C and to 35 d at 4°C. Similar observations were reported earlier. Schmidt *et al.* (1961) found that type E grew and produced toxin in beef stew within 36 d at 3.3°C. Eklund *et al.* (1967 a) had shown that increasing lengths of time were required for non-proteolytic *C. botulinum* type

B to produce toxin when the temperature was decreased and decreasing the inoculum level by 1 log increased the length of incubation required for growth and toxin production. Jensen *et al.* (1987) reported initiation of growth of type E *C. botulinum* at 20°C after 2 d, at 16° after 3-5 d, at 12°C after 6-8 d and at 8°C 11-14 d. The data reported by Jensen *et al.* (1987) correlated well with the data of the present investigation.

The lag time of *C. botulinum* type C and D isolates and the type D reference strain also increased with the lowering of storage temperature similar to type A, B and E. It increased from 2 d at 30°C to 7-8 d at 15°C. Segner *et al.* (1971 b) reported growth of marine type C strain at 15.6°C in FEM medium within 7-9 d and in ground haddock at15.6°C within 5-6 d. Similar results were observed in the present study also. The behaviour of *C. botulinum* type D isolates and the type D reference strain was similar to type C isolates. Wide strain variation with respect to size of inoculum needed to initiate growth at various temperatures has been reported for *C. botulinum* (Ohye and Scott 1953, Jensen *et al.* 1987).

The present investigation has shown that the limiting temperature permitting growth and toxin production by *C. botulinum* types A to D was 15°C and that of type E was 4°C. Growth and toxin production by *C. botulinum* is greatly influenced by the storage temperature. The minimal temperature for growth of *C. botulinum* types C and D is very near to the limiting temperature for growth of types A and B. Hence *C. botulinum* types C and D appears to pose no additional problems to the fish processing industry. Only slow growth will occur when *C. botulinum* type A and B contaminated products are kept at mild abuse temperature (15°C) and no growth at chilled storage. Only *C. botulinum* type E could initiate growth and toxin production at chilled storage. If

temperature abuse occurs, growth will be more rapid and this could also allow proteolytic strains of *C. botulinum* to grow. It is therefore necessary to include other safety factors in processed fishery products in addition to temperature to ensure that *C. botulinum* is inhibited and toxin formation cannot occur.

4.6.1.2 Effect of temperature and substrate on toxin production by *Clostridium botulinum*

Growth of *C. botulinum* type B and appearance of toxin in the culture fluid were examined over a time course of 96h. Growth was measured by plating the culture fluid in TSGYA and incubating in Gaspak jar. Maximum growth was obtained in 24h (Fig.11).

After 24h incubation at 37°C, which approximately marked the beginning of the stationary growth phase, the toxin titre of the culture filtrate was 8000 MLD/ml. Between 24h and 48h the culture lysed and after 48h the growth was resumed followed by a long stationary phase. When autolysis became significant, the toxin titres rose sharply and reached a maximum of $1.0x10^5$ MLD/ml after 72h It has been demonstrated that the toxicity of a 24h culture represented approximately 10% of the toxin found in filtrates of cultures 72h older. Comparison of the pH levels of control medium and the supernatant fluid of growing cultures showed that pH declined to a minimum of 5.5 and did not increase from this value during the 96h time period.

The amount of toxin in the culture fluid rose steadily during the first 72 h. Continued incubation for upto 96h did not further increase the toxin concentration. As shown in fig. 11, the toxin concentration in the culture fluid decreased with time after 72h When the growth and toxin curves were examined together, it is evident that the

maximum concentration of toxin was attained after lysis of the cells had occurred (fig. 11).

The effect of temperature on growth and toxin production was also examined. Growth occurred at all temperatures tested. But 37°C was apparently optimal (fig. 12A). The optimum temperature for toxin production was also found to be 37°C with maximum toxin concentrations being attained in 72h (fig. 12B).

The effect of substrate on growth and toxin production was examined at two incubation temperatures, 30°C and 37°C. The results are presented in fig. 13 & 14. Growth occurred in all the substrates tested. Maximum growth occurred in CMM at 37°C. Similarly, maximum toxin production was also noticed in CMM at 37°C followed by TPGYB and TSGYB. Variation in toxin production was also noticed. However, the overall pattern of growth and toxin production obtained with three media were essentially the same.

The relationship between bacterial growth and toxin production and conditions optimum for toxin production by *C. botulinum* were worked out earlier in static cultures (Bonventre and Kempe 1960) and later in fermenter system with control of temperature, pH, redox potential and rate of agitation and sparging (Siegel and Metzger 1979, 1980; Siegel 1981). Bonventre and Kempe (1960) attempted to correlate the kinetics of cell multiplication and subsequent autolysis with the appearance of toxin in the extracellular environment. They found a maximum toxin production of 10^4 MLD/ml in Trypticase yeast extract glucose medium (TYG) after 72h by *C. botulinum* type B in static cultures. Incubation times from 2 to 3 days upto 10 days were reported necessary to obtain comparable yields of type B toxin in static cultures (Siegel 1981).

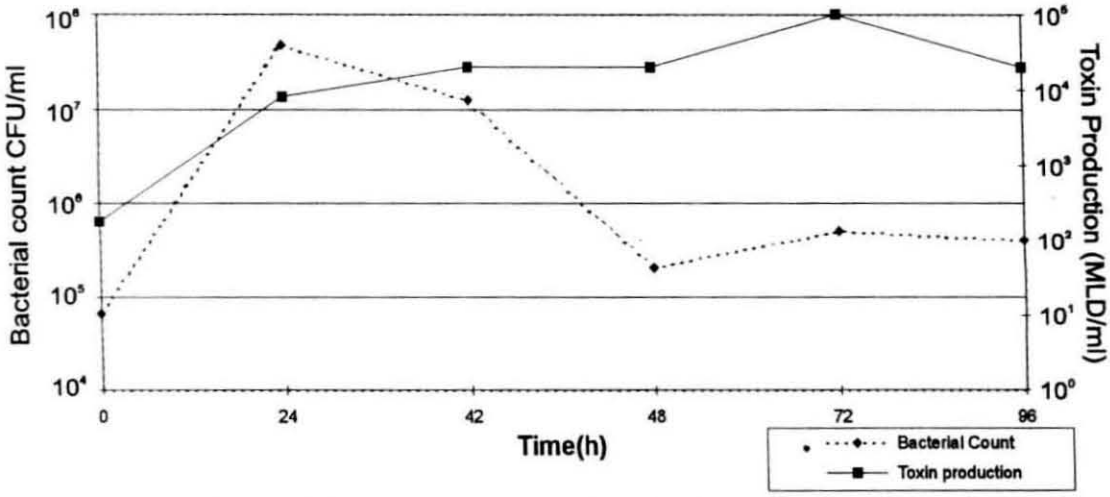

Fig. 11. Growth and toxin production of *Clostridium botulinum* type B strain 90B at 37°C

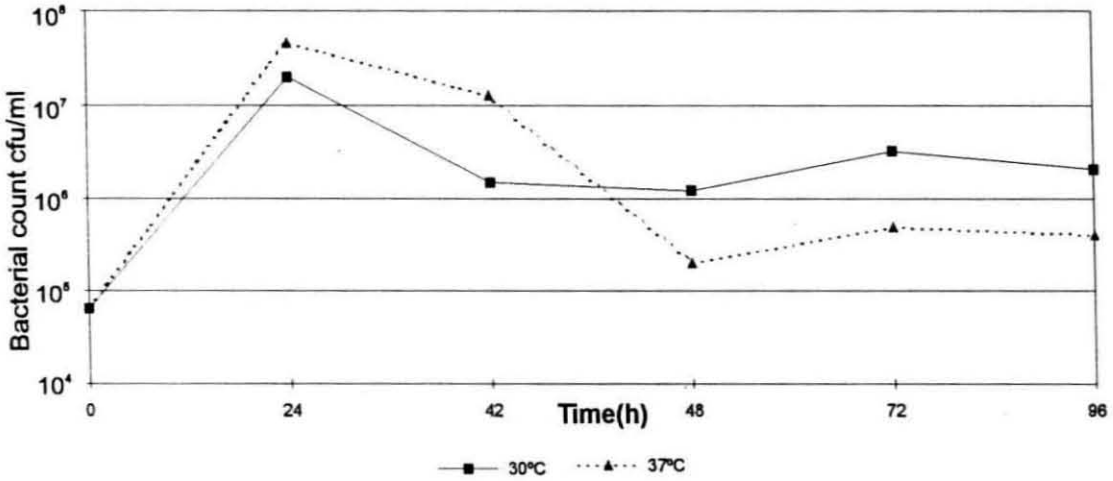

Fig. 12A. Effect of incubation temperature on growth of *Clostridium botulinum* type B strain 90B

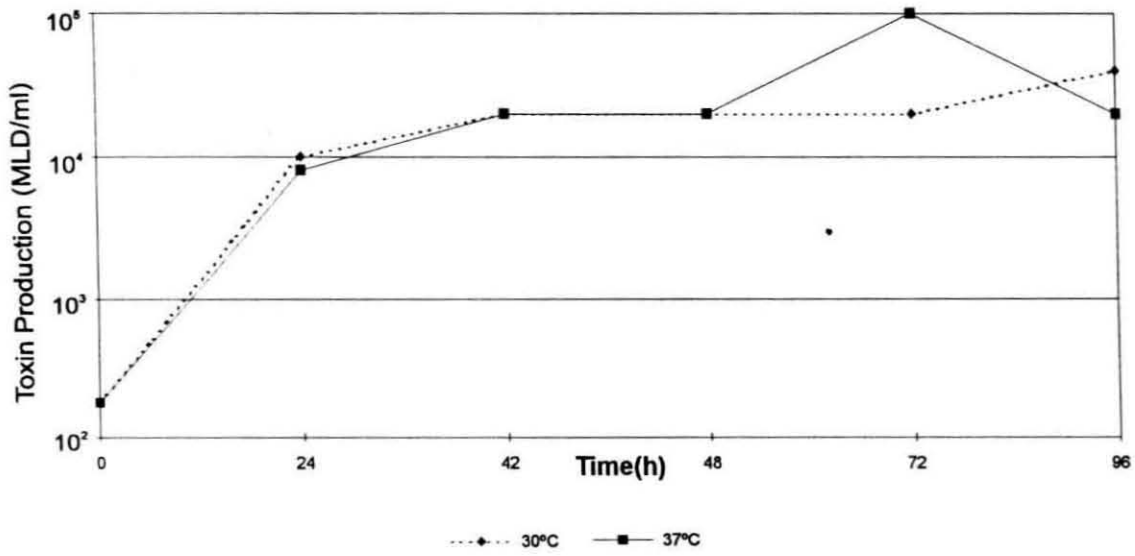

Fig. 12B. Effect of incubation temperature on toxin production of *Clostridium botulinum* type B strain 90B

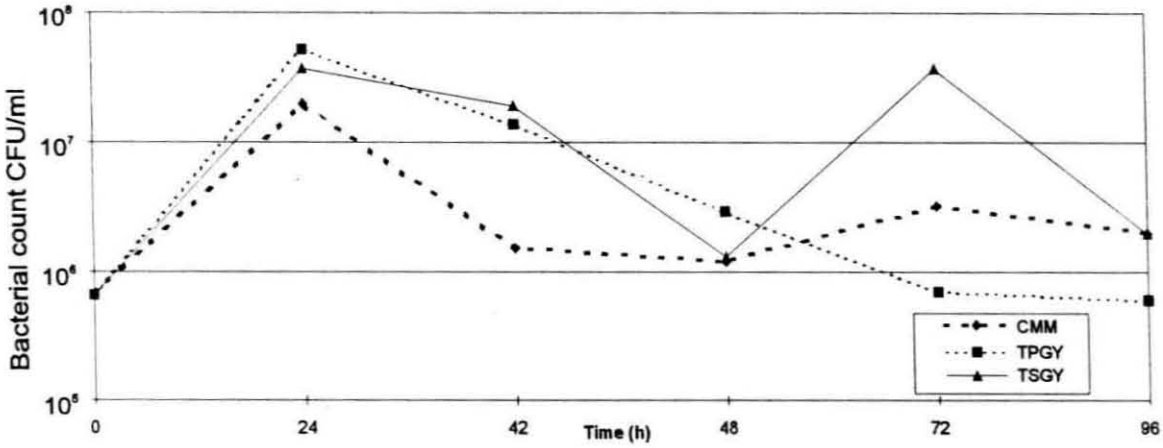

Fig. 13A. Effect of substrate on growth of *Clostridium botulinum* type B strain 90B at 30°C

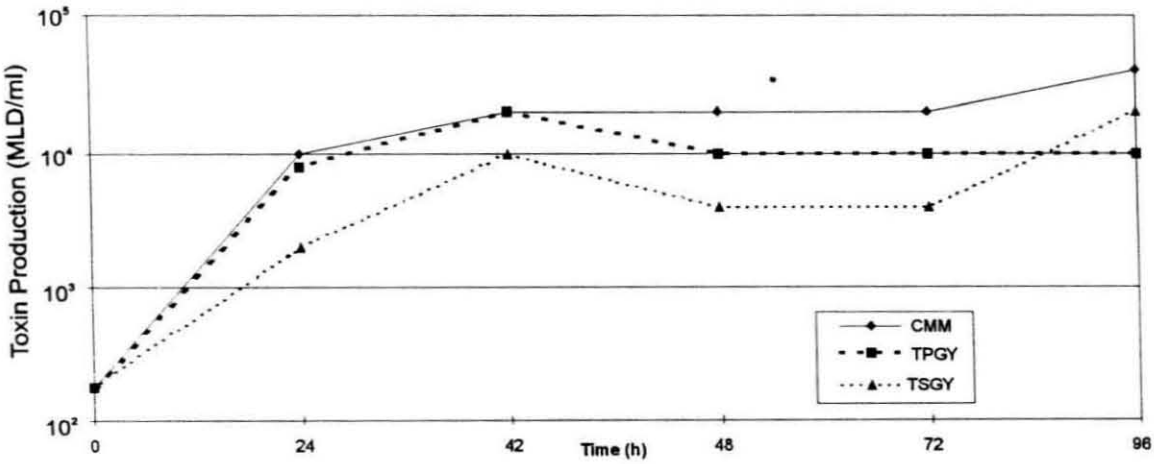

Fig. 13B. Effect of substrate on toxin production by *Clostridium botulinum* type B strain 90B at 30°C

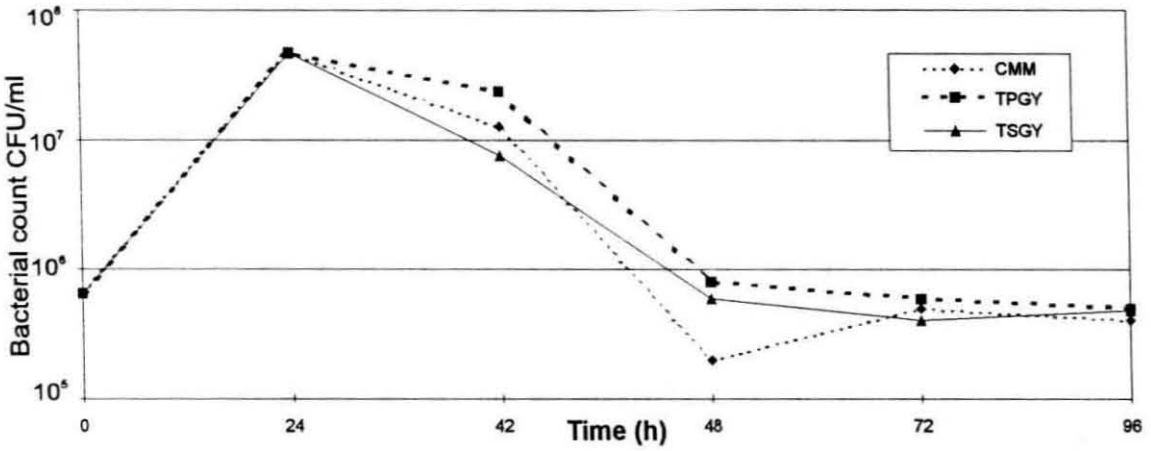

Fig. 14A. Effect of substrate on growth by *Clostridium botulinum* type B strain 90B at 37°C

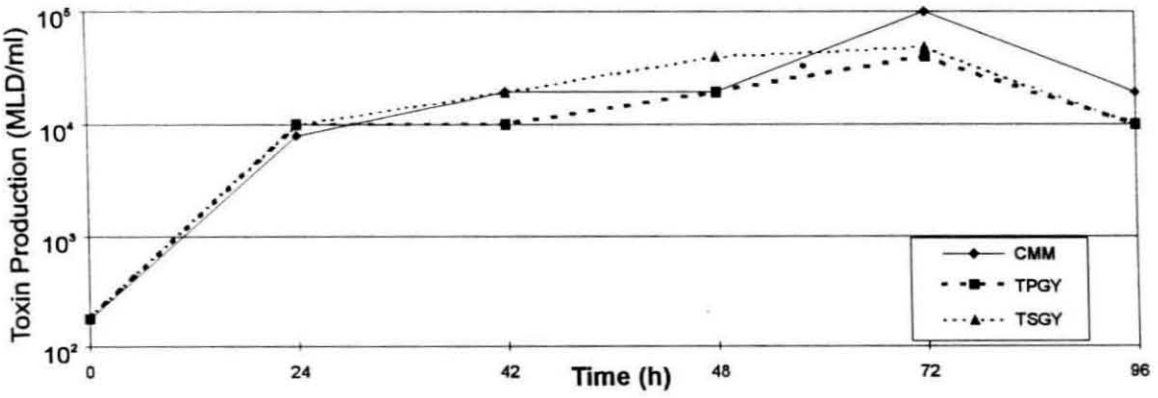

Fig. 14B. Effect of substrate on toxin production by *Clostridium botulinum* type B strain 90B at 37°C

Siegel and Metzger (1980) obtained high yields of *C. botulinum* type B toxin ($4.0x10^5$ - $5.0x10^5$ MLD/ml) at 35°C within 48h using a fermenter system. In the same study, he found okra strain of type B produced $1.0x10^6$ - $2x10^6$ MLD/ml toxin in 30h. The results of the present study agree well with that of Bonventre and Kempe (1960) in that in static cultures, maximum toxin production was noticed after 72h and maximum toxin production in TYG medium was 10^4 MLD/ml.Toxin production by *C. botulinum* type B varied in the present study depending upon the substrate used. Maximum toxin production occurred in CMM and $1.0x10^5$ MLD/ml toxin was produced whereas in TSGYB and TPGYB, maximum toxin produced was $4.0x10^4$ and $5x10^4$ MLD/ml respectively. Similar observations were reported earlier (Siegel and Metzger 1979).

Toxin titres of *C. botulinum* type B were not increased by continued incubation but it declined with time after 72h in the present study. Siegel and Metzger (1980) also reported that continued incubation for upto 96h did not further increase the toxin concentration of type B *C. botulinum* in a fermenter system but it declined after 72 h. Siegel and Metzger (1980) reported 35°C as the optimum temperature for toxin production by *C. botulinum* type B. The results of the present study also showed 37°C as the optimum temperature for toxin production.

The present investigation revealed that (1) temperature and substrate influence toxin production by *C. botulinum* type B, (2) 37°C is the optimum temperature for growth and toxin production by *C. botulinum* type B with maximum toxin concentrations being attained in 72h.

4.6.2 Influence of pH

The growth and toxin production by *C. botulinum* were influenced by the pH of the substrate (Table 22). When spores of *C. botulinum* were inoculated into CM medium type A and B isolates initiated growth and formed toxin over the pH range of 5.0-7.0 within 1-5 d. At pH 4.8, only type A isolates initiated growth and toxin formation within 12-24 d. *C. botulinum* type C isolates readily grew and produced toxin over the pH range of 5.4-7.0 within 1-5 d. At pH 5.2, growth and toxin production by *C. botulinum* type C isolates were delayed for a period of 13-26 d. Growth and toxigenesis of all *C. botulinum* type D isolates and the reference strain were noticed over the pH range 5.2-7.0 whereas at pH 5.0, only the reference strain and one isolate grew and developed toxin. However, growth and toxin production were delayed for a period of 16-17 d. *Clostridium botulinum* type E reference strain grew and developed toxin over the pH range of 5.4-7.0 within a period of 1-7 d.

The limiting pH permitting growth and toxin production by *C. botulinum* type A isolates was 4.8 whereas that of type B was 5.0. It had also been shown that pH 5.0 was the limiting pH permitting growth and toxigenesis by one type D isolate and the type D reference strain. The limiting pH permitting growth and toxin production by all type C isolates and one type D isolate was pH 5.2 whereas that of type E reference strain was pH 5.4.

It is generally considered that *C. botulinum* will not grow and produce toxin in foods at pH 4.6 or below (Ohye and Christian 1967; Ito and Chen 1978; Odlaug and Pflug 1978). Contradictory reports concerning the initiation of growth and toxin production by *C. botulinum* types A, B and E at or below pH 4.6 were made by Raatjes and Smelt

(1979), Odlaug and Pflug (1979), Smelt *et al.* (1982), Montville (1982), Tanaka (1982), Tsang *et al.* (1985), Young Perkins and Merson (1987) and Dodds (1989). Growth of *C. botulinum* at pH values 4.6 or below has usually been explained by sufficiently anaerobic growth medium, rise in pH brought about by the growth of other organisms such as bacteria, yeasts or molds, high concentration of proteins in the medium or the acidulants used. Some studies have shown that limiting pH may be higher when other conditions are suboptimal (Segner *et al.* 1966; Lund *et al.* 1985; Graham and Lund 1987).

The results of the present investigation showed that spores of *C. botulinum* type A isolates survived at acid pH and growth and toxin production occurred at pH 4.8 whereas type B was inhibited at pH 4.8. The limiting pH permitting growth and toxin production by type A was higher than that reported in the earlier investigations. This variation in the limiting pH reported in the present study may be attributed to the variation in the substrates used, the variability in acid tolerance exhibited by different strains of *C. botulinum* and the anaerobic conditions in the growth medium as shown by the previous studies. (Huhtanen *et al.* 1976; Ito and Chen 1978; Smelt *et al.* 1982 and Tsang *et al.* 1985; Young Perkins and Merson 1987). The limiting pH, permitting growth and toxin production by *C. botulinum* type B, reported in the present study agrees well with that reported earlier (Baird-Parker and Freame 1967; Ito and Chen 1978; Odlaug and Pflug 1979 and Smelt *et al.* 1982). However, growth and toxin production by *C. botulinum* type B in the pH range 4.77-4.91 have been reported earlier (Lund *et al.* 1985). Tanaka (1982) reported growth of strains of *C. botulinum* type A and B at pH values of 4.24 to 4.4.

Table 22: Influence of pH on growth and toxin production by *Clostridium botulinum*

pH of the substrate (CMM)	*C. botulinum* outgrowth time in d (range in parenthesis)								
	53A	131A	90B	18C	275C	2693D	ATCC 27517	2131 D	NCIB 10660
	$3.4x10^6$ spores	$2.0x10^6$ spores	$3.2x10^6$ spores	$3.1x10^6$ spores	$6.0x10^5$ spores	$1.0x10^6$ spores	$7.6x10^7$ spores	$6.0x10^6$ spores	$4.2x10^6$ spores
7.0	1	1	1	2	2	2	2	1	1
6.0	2	2	2	2	4(3-5)	7(4-8)	2(2-3)	2	3(3-5)
5.8	5(4-7)	4(3-8)	2(2-4)	2(2-5)	6(5-9)	7(5-9)	2(2-5)	5(4-8)	4(3-6)
5.6	5(4-9)	4(3-7)	5(4-9)	5(4-9)	6(5-9)	7(6-9)	5(4-9)	5(4-9)	5(4-9)
5.4	5(4-9)	4(3-8)	5(4-9)	5(4-9)	6(5-10)	8(6-10)	5(4-9)	5(4-9)	8)6-12)
5.2	5(4-9)	4(3-8)	5(4-9)	13(11-16)	26	8(6-12)	5(4-9)	5(4-9)	-
5.0	5(4-10)	8(6-10)	5(4-9)	-	-	-	17(16-21)	16(15-24)	-
4.8	24(21-29)	12(11-19)	-	-	-	-	-	-	-
4.6	-	-	-	-	-	-	-	-	-

Segner *et al.* (1966) found the minimum pH supporting growth of type E in a variety of culture media to be in the range of 5.24-5.36 whereas Dolman and Iida (1963) and Tsang *et al.* (1985) had shown growth and toxin production by *C. botulinum* type E in the pH range 4.0-4.3. The limiting pH permitting growth and toxin production by type E reference strain was 5.4 in the present study. The results showed good agreement with that of Segner *et al.* (1966). The variation in the limiting pH observed in the present study may be attributed to the variation in the substrate and the bacterial strain involved and the strict anaerobic conditions used as shown by Ito and Chen (1979), Smelt *et al.* (1982) and Tsang *et al.* (1985).

It has been shown that the limiting pH, permitting growth and toxin production by marine strains of *C. botulinum* type C was 5.1 (Segner *et al.* 1971 b). One strain however, showed a limiting pH of 5.25. Variability to acid tolerance has been reported for strains of *C. botulinum*. Huhtanen *et al.* (1976), Ito and Chen (1978). Emodi and Lechowich (1969 a) showed minor variations in acid tolerance among type E strains. Similar variation in acid tolerance is exhibited by *C. botulinum* type C and D strains in the present study. The variations in the limiting pH among strains of type D may be attributed to the different rates of germination in suboptimal condition by *C. botulinum* spore crops. Lund *et al.* (1990) have stated that different spore crops of the same strain of *C. botulinum* can show dissimilar properties in particular different rates of germination in sub optimal condition. The limiting pH permitting growth and toxin production by *C. botulinum* type C and D isolates, in general was in the pH range 5.0-5.2. The results of the present study agree well with that of Segner *et al.* (1971 b). Data on the limiting pH of *C. botulinum* type D is very limited. Because the properties of type C strains are, in

general, similar to those of type D and both are grouped together in Group III by Sneath (1986), it is to be expected that the limiting pH permitting the growth and toxin production by type D is very near to that accepted as limiting for growth of type C strains.

It has been demonstrated in the present investigation that inhibition of *C. botulinum* increases as the pH decreases from 7.0-4.6. A decrease in pH from pH 7.0 to pH 4.8 resulted in progressively longer delays before growth and toxin production. At 4.8, the growth toxin production by *C. botulinum* type A was delayed for a period of 12-24 d. Several earlier investigations had also shown the influence of pH on the inhibition of *C. botulinum* (Baird Parker and Freame 1967; Ohye and Christian 1967; Sperber 1982; Dodds 1989). Lund *et al.* (1987) have reported that a decrease in pH below 4.9 resulted in a marked decrease in the probability of growth of vegetative cells of *C. botulinum*. Dodds (1989) reported growth and toxin production by *C. botulinum* type A at pH value 4.83 and a_w 0.977 within 35 d. There are several published reports of growth at a pH below 5.0 within 14 d or less from spores of *C. botulinum* (Tanaka 1982). Similarly, it was shown in the present investigation that a decrease in pH below 5.4 and 5.2 resulted in progressively longer delay before growth and toxin production by *C. botulinum* type C and D isolates. Time intervals for growth and toxin production by *C. botulinum* type A over the pH range 4.8-7.0 were generally similar to that by type B. Similarly time intervals for growth and toxin production by *C. botulinum* type C were generally similar to that by type D in the present study. Baird-Parker and Freame (1967) have shown growth and toxin production by *C. botulinum* type A and B at 30°C and pH 5.3 within 5 d. This may be attributed to the cultural similarities of these two strains.

The results of the present study revealed that *C. botulinum* type A and proteolytic

strains of type B are potentially capable of growing in culture media at pH values between 4.8-5.0 whereas type C, D and E strains are potentially able to grow at pH values between 5.0-5.4 in a period of few weeks. Outbreaks of botulism involving acidified products were reported in the past (McClure *et al.* 1994, Hauschild 1993). Therefore, the potential for growth and toxin production by *C. botulinum* in acidified conditions must now be recognized.

The present study showed the limiting pH permitting growth and toxin production by *C. botulinum* type A to be 4.8, type B to be 5.0, type C and D to be 5.0-5.2 and type E to be 5.4. Minor variations in acid tolerance was noticed among strains of *C. botulinum* types C and D. Inhibition of growth of *C. botulinum* increased as the pH decreased from 7.0-4.6. The study shows that growth and toxin production by *C. botulinum* types A and B are not prevented at acid pH. Therefore, it is suggested that other safety factor should be included in addition to acidification to prevent growth and toxin production by *C. botulinum* in processed fishery products. An understanding of the mechanism by which an acidulant exerts its inhibitory effect either alone or in combination on *C. botulinum* is of great importance in establishing hazards associated with consumption of acid foods.

4.6.3 Influence of water activity (a_w)

The effect of decreasing water activity (a_w) through variation in sodium chloride concentration on growth and toxin production in CMM at pH 7.0 was shown in table 23. Addition of salt decreased the water activity from 0.985 (control) to 0.919 for medium containing 12% salt. The addition of salt and reduction in a_w of the medium had a significant effect on growth and toxin production by *C. botulinum*. In control (a_w 0.985)

medium, toxin was detected within 1-2 d by all strains of *C. botulinum* tested.

At 30°C, medium containing 3% sodium chloride (a_w 0.976) became toxic within 2 to 5 d when inoculated with *C. botulinum* type A and B isolates and the reference strains whereas type C and D isolates and type D and E reference strains grew and produced toxin within 2-3 d. However, at 4% salt concentration (a_w 0.972) growth and toxin production by type E reference strain was prevented while only a progressive delay in toxin production was noticed by all the other strains of *C. botulinum*.

Growth and toxin production by one isolate each of type C and D were prevented when the salt concentration was increased to 5% (a_w 0.97) while others grew and produced toxin within 5-8 d. However, at salt concentrations greater than 5%, growth and toxin production was not detected in any tubes inoculated with *C. botulinum* type C, D and E throughout the 35 day of storage. Sodium chloride at concentration 6% (a_w 0.962) delayed growth and toxin production of *C. botulinum* type A and B isolates and the reference strains for a period of 6-9 d. *C. botulinum* types A and B isolates and the reference strains initiated growth and toxin production when the salt concentration was increased to 8% (a_w 0.952). But a progressive delay in toxin production was observed for a period of 11-13 d.

At salt concentrations greater than 8%, i.e. 10% and 12%, toxin was not detected throughout 35 day of storage at 30°C. The decrease in a_w obtained by adding salt delayed toxin production at higher salt levels.

The minimum a_w permitting growth and toxin production by type E reference strain was 0.976, for type C and D it was 0.97 and for type A and B it was 0.952 in the present study. These values corresponded to sodium chloride concentration of 3%, 5% and 8% respectively.

Table 23: Influence of water activity on growth and toxin production by *Clostridium botulinum*

a_w value	% Sodium chloride w/w	*Clostridium botulinum* outgrowth time in days (range is shown in parenthesis)										
		53A	131A	Type A NCIB 10640	9B	Type B NCIB 10657	18C	275C	2131D	2693D	Type D ATCC 27517	Type E NCIB 10660
		$3.4x10^4$	$8.5x10^5$	$2.7x10^6$	$3.2x10^5$	$1.5x10^5$	$3.1x10^6$	$3.0x10^7$	$3.0x10^7$	$1.1x10^5$	$1.7x10^5$	$9.0x10^4$
0.985	0	1	1	1	1	1	1	1	2	1	2	1
0.976	3	2	4	2	2	5(3-6)	2	2	3	3	3	3
0.974	4	6(5-8)	4(3-6)	5(3-6)	6(3-7)	8.6-9)	6(3-7)	4	4	6(3-7)	6(3-7)	-
0.97	5	6(7-8)	6(4-7)	5(4-7)	6(3-7)	8(6-9)	6(3-7)	-	-	6(3-7)	8(6-9)	-
0.962	6	8(7-10)	8(7-9)	6(5-8)	8(6-9)	9(8-11)	-	-	-	-	-	-
0.952	8	13 (11-14)	12 (11-13)	12 (11-14)	13 (11-15)	13 (11-15)	-	-	-	-	-	-
0.936	10	-	-	-	-	-	-	-	-	-	-	-
0.919	12	-	-	-	-	-	-	-	-	-	-	-

The effects of sodium chloride on growth of *C. botulinum* have received considerable attention in the past. In general, studies conducted over the last fifty years indicate that when all other conditions are favourable in the substrate, 5% sodium chloride is required for prevention of growth and toxin production of non-proteolytic strains and 10% for proteolytic strains and these salt concentrations correspond to a_w 0.97 and 0.94 respectively (Sperber 1982; McClure *et al.* 1994). The results of the present study indicated that *C. botulinum* type E possesses a level of salt tolerance considerably below that recognized for type A and B as shown in the previous studies.

At salt concentrations greater than 8%, neither growth nor toxin production by types A and B were noticed in the present study. In a review on the differences and similarities of proteolytic and non-proteolytic *C. botulinum* strains, Lynt *et al.* (1982) stated that at salt concentrations of 8.6-10.5%, growth of *C. botulinum* types A and B was prevented. Similarly Scott (1957) has shown that 8% sodium chloride permitted growth of *C. botulinum* whereas levels above this inhibited growth. Similar results were noticed in the present study. In contrast, Ohye and Christian (1967) reported that 9.4% sodium chloride concentration permitted growth of *C. botulinum* types A and B. The minimum a_w permitting growth of *C. botulinum* has been shown to be influenced by incubation temperature, strains tested, solute type etc. by many investigators (Baird-Parker and Freame 1967; Emodi and Lechowich 1969b; Segner *et al.* 1966; Mc Clure *et al.* 1994). The difference in the minimum a_w values for growth of *C. botulinum* types A and B as observed in the present study may be attributed to the variations in the strains and the

variation in the germination rates of different spore crops of the same strains under sub optimal conditions.

Many investigations have been made to determine the effect of water activity and sodium chloride concentration on the growth and toxigenesis of *C. botulinum* (Scott 1957; Baird-Parker and Freame 1967; Ohye and Christian 1967; Marshall *et al.* 1971) and several reviews have been published (Sperber 1982; Gaze 1992; Mc Clure *et al.* 1994). Scott (1957) had shown the minimum a_w permitting growth of proteolytic strains of *C. botulinum* to be 0.95 whereas Ohye and Christian (1967) have shown it to be 0.94. The results of the present study agrees with that of Scott (1957) in that 0.952 was the limiting water activity permitting growth of proteolytic strains of *C. botulinum* type A and B. The present finding that 0.936 prevented growth and toxin production by proteolytic strains of *C. botulinum* is consistent with that of Ohye and Christian (1967).

Certain strains of type E failed to grow in 4.5% sodium chloride whereas others were able to tolerate 4.8% sodium chloride (Segner *et al.* 1966; Baird-Parker and Freame 1967; Ohye and Christian 1967; Emodi and Lechowich 1969b; Roberts and Ingram 1973). Segner *et al.* (1966) and Emodi and Lechowich (1969b) used high inoculum of type E spores in their studies.. Emodi and Lechowich (1969b) reported variation among type E strains in salt tolerance. Mc Clure *et al.* (1994) have indicated that strains tested and incubation temperature have influence on the sodium chloride concentration required to inhibit growth. The growth and toxin production by type E were prevented at 4% sodium chloride concentration in the present study. The variation in the result may be attributed to the variation in strain and in the rate of germination of spores. Variation in the rate of germination of spores of different spore crops of the same strain under sub

optimal conditions has already been reported (Roberts and Thomas 1982; Lund *et al.* 1990). The inoculum used in the present study is very low compared to the above studies. Therefore, the number of spores germinating in high salt concentration will also be low.

Emodi and Lechowich (1969b) found the minimum a_w permitting the growth of type E to range from 0.972 to 0.978 in a wide variety of solutes. The minimum a_w value for growth of type E in the present study falls within this range. It has also been demonstrated in this study that type E is much more sensitive to salt than type A and B as shown in the previous studies (Segner *et al.* 1966).

Segner *et al.* (1971 b) investigated the sodium chloride sensitivity of marine strains of *C. botulinum* type C. They found that marine strains were tolerant to 2% sodium chloride and one strain grew in 2.5% salt. They have also shown variation among strains in salt tolerance. Similar variations in salt tolerance among type C and D strains were observed in the present study. Contrary to the above findings of Segner *et al.* (1971 b) all type C isolates in the present study showed tolerance upto 4% salt and one isolate showed tolerance upto 5% (a_w 0.97). Similarly type D reference strain and one type D isolate also grew and produced toxin in 5% salt while others showed tolerance upto 4% only. There appear to be no published data on the salt inhibition of type D spores. Because *C. botulinum* type D strains are culturally similar to type C strains and they are grouped together (Sneath 1986), the minimum a_w permitting growth of type C may also permit growth of type D isolates as shown in the present study.

The present study revealed that the minimum water activities permitting growth of *C. botulinum* type A and B spores types C and D spores and type E spores in sodium

chloride containing media were found to be 0.952, 0.97 and 0.976 respectively. These values corresponded to sodium chloride concentrations of 8%, 5% and 3% respectively. Variations in salt tolerance were observed among type C and D strains. *C. botulinum* type E possessed a level of salt tolerance considerably below that recognized for types A and B. The study indicates that salt exert antibotulinal effects, resulting in inhibition of growth and toxin production of *C. botulinum* spores. The study points out that 10% sodium chloride is adequate for complete botulism control. Increasing the salt concentration of fishery product to such a level would minimise their acceptance by consumer. Therefore, the possibility of using salt concentrations considerably lower than 10% must be explored by combining other factors such as pH, temperature etc. to exhibit sufficient inhibitory activity against botulinal growth.

4.6.4 Effect of gaseous atmosphere

4.6.4.1 Growth and toxin production of *Clostridium botulinum* in mullet tissue homogenate stored under vacuum

All the inoculated mullet tissue homogenate held at 30°C under vacuum became toxic within 2 d (table 24). Storage of inoculated samples at moderate abuse temperature (15°C) progressively delayed toxin production for a period of 5 d and toxin was detected on 6th day in packs inoculated with *C. botulinum* types A and B strains and on 8th day in packs inoculated with types C and D. The reduction in storage temperature to 15°C inhibited toxin production by *C. botulinum* type E upto 2 d. Neither growth nor toxin production was noticed in the control pack.

Lowering the storage temperature of the inoculated packs to 10°C inhibited outgrowth and toxigenesis of all types of *C. botulinum* except type E. Storage of

inoculated vacuum packed samples at 10°C delayed growth and toxin production of type E upto 8 d. Storage at refrigeration temperature (4°C) completely inhibited toxin production by types A, B, C and D. Following the transfer of packs from 4° and 10°C to 30°C after 40 d storage, growth and formation of toxin occurred within 5 d. Type E was capable of growth at 4°C. Outgrowth and toxin production was observed on 28th day in mullet tissue homogenate stored at 4°C under vacuum.

The amount of toxin produced in mullet-tissue homogenate inoculated with *C. botulinum* type A to E at 4°C to 30°C was shown in fig.15. Toxin was detected in the inoculated packs stored at 30°C on the first day. The toxin titres were 10000, 5000, 60, 80 and 1000 MLD/10g for *C. botulinum* types A, B, C, D and E respectively. During storage at 15°C, toxin was not detected in any sample during the first two d. On day 3, toxin titres were 100 MLD/10g in types E inoculated packs and on day 6, toxin titres were 100 MLD/10g in types A and B inoculated packs and on day 8, toxin titres were 8 MLD/10g in type C inoculated packs and 6 MLD/10g and 8MLD/10g in type D inoculated packs. Toxin was not detected in any packs upto 7 d during storage at 10°C. On day 8, toxin titres of 80 MLD/10g was noticed in packs inoculated with type E. At 10°C neither growth nor toxin production was noticed in packs inoculated with types A, B, C and D. Toxin developed after 27 d in mullet tissue homogenate inoculated with type E and stored at 4°C. Toxin titres of 10 MLD/10g were developed.

Table 24: Growth and toxin production in mullet tissue homogenate inoculated with *Clostridium botulinum* and packed under vacuum

C. botulinum Strain	Inoculum level per 10g	Storage temperature (°C)	Toxin detection (d)
53A	$7.2x10^5$	30	1
		15	6
		10	-
		4	-
90B	$1.3x10^5$	30	1
		15	6
		10	-
		4	-
18C	$1.6x10^6$	30	2
		15	8
		10	-
		4	-
93D	$5.0x10^5$	30	2
		15	8
		10	-
		4	-
ATCC 25717 Type D	$1.5x10^6$	30	2
		15	8
		10	-
		4	-
NCIB 0660 Type E	$1.0x10^3$	30	1
		15	3
		10	8
		4	28

The pH of the mullet tissue homogenate increased from an initial value of 5.8 to 6.7 in types A and B inoculated packs and from 5.8 to 6.2 in types C and D inoculated packs. There was no change in pH in type E inoculated packs.

Strasdine and Kelly (1967) found rapid germination of type E spores in the extracts of cod, salmon, sole and herring. Boyd and Southcott (1968) compared toxin production in cod, lemon sole and Coho salmon and found cod flesh to be superior in

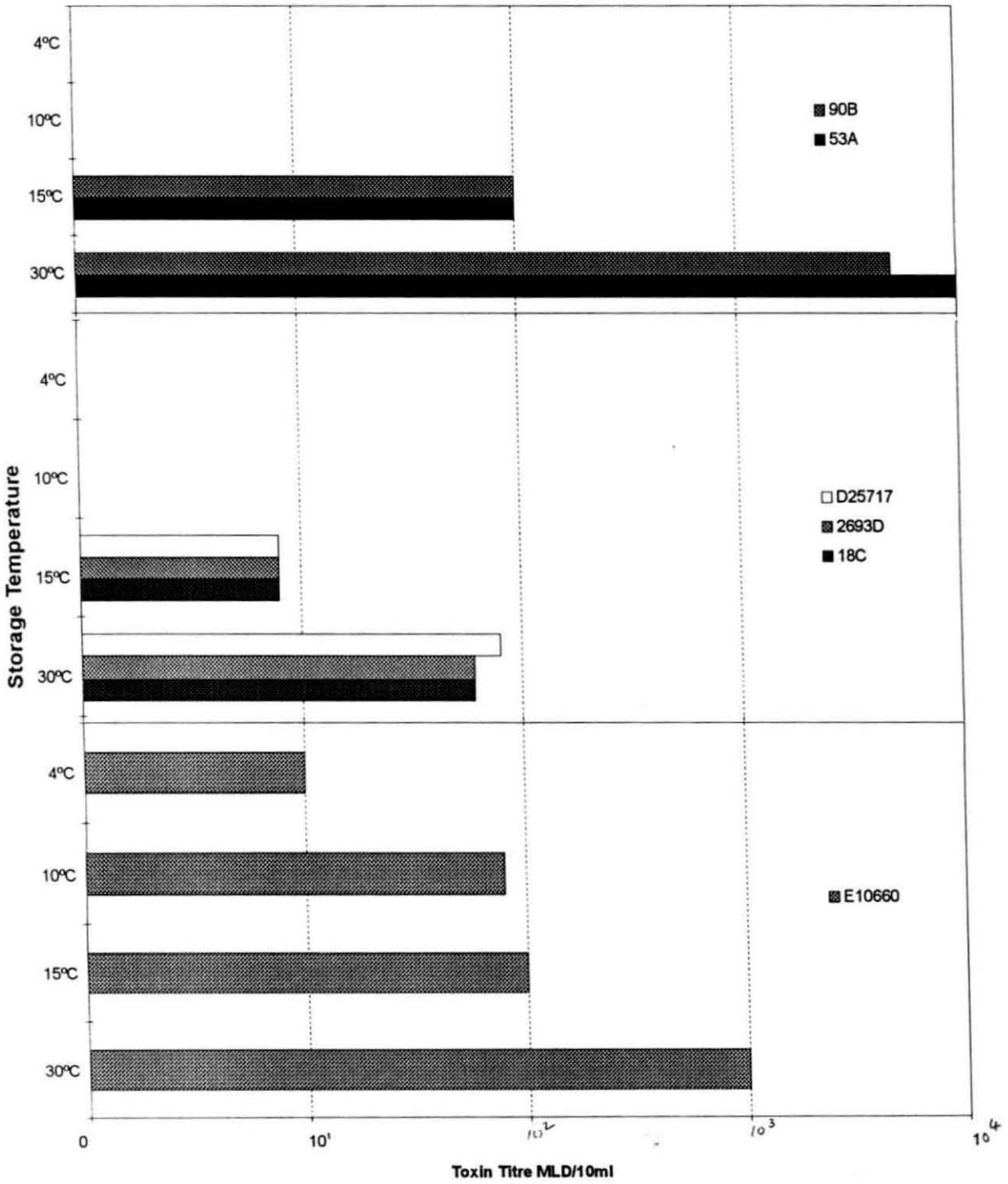

Fig. 15 Comparison of toxin production by *Clostridium botulinum* type A to E in mullet tissue homogenate

supporting growth. It was demonstrated earlier that dead fish provide an excellent medium for growth and toxin production of *C. botulinum* and the oil content of fish flesh has been suggested as a criteria for evaluating the botulinisity between fish species (Abrahamsson *et al.* 1965; Cann *et al.* 1965; Huss 1981). Baker and Genigeorgis (1990) found that rock fish and salmon seemed to be more conducive to *C. botulinum* growth and toxigenesis than sole tested. Eyles and Warth (1981) and Eklund (1982) while reviewing the preservation by Modified Atmosphere and the safety of these processes stated that it is necessary to determine whether *C. botulinum* can grow and produce toxin in food samples to evaluate the potential public health hazards of this method of preservation. In the present study, mullet tissue offered a balance nutrient for the growth *C. botulinum* on account of its high fat content compared to shrimp and supported growth and toxin production by *C. botulinum* types A to E. But the rate of toxin production by types C and D was less compared to types A, B and E. The study indicated that mullets are botulinogenic in many respects i.e., initial contamination, growth potential and toxin stability and therefore a potential health hazard exists if modern methods of preservation such as vacuum/Modified Atmosphere packaging are adopted.

The minimum temperature that allowed growth and toxin production by types A, B, C and D was 15°C in the mullet tissue homogenate. Similar observations were made earlier. Garcia and Genigeorgis (1987) have already demonstrated toxigenesis in salmon tissue homogenates stored under vacuum by the residential spores of *C. botulinum* type A at temperatures 16° and 30°C but not at or below 12°C. Segner *et al.* (1971 b) found growth and toxin production by type C in ground haddock and the minimum growth

temperature of marine strains of *C. botulinum* type C was found to be 15.6°C. The results of the present study are in agreement with the earlier report (Segner *et al.* 1971b) that the minimal temperature for growth of *C. botulinum* type C is very near to that accepted as limiting for growth of type A and proteolytic type B.

The limiting temperature for growth and toxin production by type E was shown to be 4°C in the present study and the toxin production was delayed for a period of 28 d. It was demonstrated earlier that type E grew and produced toxin at 3°C and 4°C. Studying the growth and toxin production by *C. botulinum* type E in vacuum packed fish, Cann *et al.* (1965b) showed that herring became toxic after storage at 5°C for 15 d under vacuum with as small an inoculum as 10^2 spores/pack. In the same study, they were also able to demonstrate toxin production in whole vacuum packed herring inoculated with 100 spores/ pack and stored at 3.3 and 5°C on 21st and 9th day respectively. Lindroth and Genigeorgis (1986) reported growth and toxin production by non-proteolytic *C. botulinum* types B, E, F in inoculated red snapper tissue homogenates stored under vacuum on 1st day at 30°C, 3rd day at17°C, 6th day at 12°C and 21st day at 4°C. In a similar study, Garcia and Genigeorgis (1987) reported that the shortest lag phase for the non-proteolytic *C. botulinum* types in salmon tissue homogenates stored under vacuum was 12 day at 4°C for 10^4 spore inoculum and 18 d for 10^3 spore inoculum. In all of the above studies, only type B toxin was found at 4° and they suspected that this inhibition of type E in the presence of type B may be due to boticins. Reddy *et al.* (1996) did not find toxin development by type E in fresh tilapia fillets during storage of 90 d at 4°C under vacuum. Inhibition of growth of type E in tilapia fillets at 4°C may be attributed to the

low fat content. Abrahamsson *et al.* (1965) and Cann *et al.* (1965 b) had reported that fish with a high fat content provide a better medium than non fatty fish. Sperber (1982) had shown that a particular substrate may be able to exert an inhibitory influence as the minimum growth temperature is approached.

Garcia and Genigeorgis (1987) calculated a lag period (LP) of 4 d using the prediction model for salmon tissue homogenates inoculated with 10^4 non-proteolytic *C. botulinum* spores/sample and stored at 10°C under vacuum while Lindroth and Genigeorgis (1986) and Ikawa and Genigeorgis (1987) estimated LP for rock fish tissue homogenates to be 4.07 d and 4.49 d respectively under the same conditions. Baker and Genigeorgis (1990) while evaluating the toxigenesis of non-proteolytic *C. botulinum* in rock fish stored under modified atmospheres by predictive models estimated the lag times of 14.29 d and 6.32 d for 3.3 and 5°C respectively. The results of the present study agree well with that of Ikawa and Genigeorgis (1987) in that the lag period for mullet tissue homogenates inoculated with type E was found to be 5 d at 10°C.

Storage temperature of mullet tissue homogenate had an influence on the toxin titres of *C. botulinum*. Type A and B strains produced 10000 MLD/10 g and 5000 MLD/10 g respectively at 30°C and 100MLD/10 g at 15°C. Similarly type C and D strains produced toxin titres of 60 and 80 MLD/10 g respectively at 30°C and 8 and 6 MLD/10 g respectively at 15°C. Type E strains developed toxin titres of 1000 MLD/10g and 100 MLD/10g, 80 MLD/10g and 10 MLD/10g respectively at 30°C, 15°C, 10°C and 4°C. Similar observations were recorded by other workers also. It has been demonstrated earlier that herring inoculated with 10^2 spores/g developed four times the MLD/g as

halibut inoculated similarly and stored at 20°C, about twice the MLD/g at 10°C while less than 10 MLD/g developed in each substrate at 5°C. However, the time to initial toxicity was approximately the same for each fish regardless of incubation temperature (Cann *et al.* 1967). Huss and his co-workers (1979a, b) have shown that the storage temperature and the spore load in fish markedly influence the toxin titres of type E in vacuum-packed fish. They found toxin titres 500 MLD/g after 1 d at 30°C and at 15°C, 50 MLD/g toxin was detected after 3 d. The present study confirms the earlier findings.

The significance of storage temperature on growth and toxin production by *C. botulinum* was demonstrated in this study. *C. botulinum* types A, B, C and D did not grow at 10°C or below in mullet tissue homogenate while *C. botulinum* type E grew at 10°C and 4°C. By transferring the packs stored at 10°C and 4°C to 30°C, growth and toxin production by *C. botulinum* types A, B, C and D were noticed within 5 d. This finding is in agreement with the earlier reports (Cann *et al.* 1965b, 1966; Huss *et al.* 1979 a) which emphasised the significance of storage temperature and to a lesser degree, the number of spores present in fish on the growth and toxin production by *C. botulinum* type E. Rapid toxin production by type E was noticed after storage for 1 d at 30° and a further reduction in storage temperature (15°C) delayed toxigenesis for 2 d and lowering the storage temperature to 10°C toxin production was detected after 8 d. Baker and Genigeorgis (1990) also reported that temperature is the most important variable contributing the greatest effect on *C. botulinum* growth and toxigenesis and initial spore load was the second most variable in Modified Atmosphere packed fresh fish.

In the present study, the maximum level of *C. botulinum* found in fish was

70/100 g. Non-proteolytic *C. botulinum* strains (BEF) were not encountered in fish (4.1.1). However, it is to be expected that non-proteolytic *C. botulinum* strains may be present in fish. The lag time observed for mullet tissue homogenate was 6d at 10°C for 10^3 spore inoculum. Ikawa and Genigeorgis (1987) have shown that by reducing the spore inoculum from 10^4 to 10^2 i.e. hundred fold reduction in rock fish tissue homogenates stored at 10°C, the lag phase was increased from the 4.49 d to 7.59 d i.e. it just doubled. Therefore, by reducing the spore level of the present study from 10^3 to 7 i.e. more than hundred fold reduction, the lag phase could be further increased from 6 d to > 12 d in mullet tissue according to the estimation of Ikawa and Genigeorgis (1987). It also indicates that fish fillets packed under vacuum can be stored safely upto 12 d at 10°C. Genigeorgis (1985) has also indicated that due to lower incidence of psychrotrophic microorganisms in the tropics, fish from the tropics are more stable at refrigeration temperatures and fish spoilage may be delayed and follow toxigenesis. Reddy *et al.* (1992) have stated that vacuum packaged fishery products held at severe temperature abuse conditions may pose a public health hazard because toxin formation coincided with sensory spoilage.

The results of the present investigation indicated that storage temperature of fish marketedly influence growth and toxin production of *C. botulinum*. The naturally occurring levels of *C. botulinum* in fish will not initiate toxigenesis in vacuum packed fish at 4°C for upto 21-25 d. If strict temperature control cannot be guaranteed, the storage time has to be limited. The study points out that if non-proteolytic *C. botulinum* spores are present in fishery products, even proper refrigeration may not be sufficient as

these strains may grow at 4°C. Mullet tissue on account of its high fat content provided a good substrate for growth and toxin production by *C. botulinum* types A to E and therefore a potential health hazard does exist, however, if such products are stored above 4°C while in modified atmospheres. Another implication of the present finding is that dead fish in fish farms provide an excellent substrate to support growth of *C. botulinum*. Proper hygiene in fish farms including such measures as frequent removal of dead fish from ponds is recommended to keep the contamination of farmed fish at a low level. The extent to which fresh/frozen fish are processed to increase shelf life or to prepare new products and the conditions under which they are stored therefore have a marked effect on the potential problems from *C. botulinum*.

4.6.4.2 Growth and toxin production by *Clostridium botulinum* in shrimp tissue homogenates stored under vacuum

Growth and toxin production by *C. botulinum* types A to E in shrimp tissue homogenates stored under vacuum at temperatures 30°C, 15°C, 10°C and 4°C were examined. Results are summarised in table 25. Upon incubation at 30°C, all the packs inoculated with *C. botulinum* types A, B, C, D and E became toxic after 24 h. There was no toxin production in the control pack. A progressive delay in toxin production was observed when the packs were incubated at a lower temperature (15°C). Toxin was detected in packs inoculated with *C. botulinum* types A and B after 5 d, in packs inoculated with types C and D after 7 d whereas packs inoculated with type E developed toxin after 4d.

Storage at mild abuse temperature (10°C) and refrigeration temperature (4°C) completely inhibited growth and toxin production by *C. botulinum* types A, B, C and D

in shrimp tissue homogenates. But when the packs were transferred to 30°C, toxigenesis was noticed after 6 d. Storage of inoculated packs under vacuum at 10°C delayed growth and toxin production by type E upto 8 d and toxin was detected after 9 d. At 4°C, toxin was developed in type E inoculated packs after 34 d.

Toxin titres produced at various temperatures by *C. botulinum* types A to E were also estimated (Fig. 16). During storage at 30°C, toxin developed after 24 h and on the second day the toxin titre was 8000 MLD/10g. for type A strain and 10000 MLD/10g for type B strain. On the other hand, toxin titres of type E was less than 20 MLD/10g. Toxin titres of 500 MLD/10g were developed in type C inoculated packs, 600 MLD/10g in type D type cultures (ATCC 27517) inoculated packs and 50 MLD/10g in packs inoculated with type D strain isolated from fish. At 15°C, toxin titres of 100 MLD/10g were found in types A and B inoculated packs on day 6 and 8 MLD/10g in type E inoculated packs on day 5. Type C, type D type culture (ATCC 27517) and type D strain from fish developed toxin titres of 20, 30 and 8 MLD at 15°C. Only type E grew and produced toxin during storage at 10°C and 4°C and toxin titres of 8 and 6 MLD respectively were developed in shrimp tissue homogenate.

The pH increased from an initial value of 6.2 to 7.0 in types A and B inoculated shrimp tissue homogenate and from 6.2 to 6.7 in types C and D inoculated packs. There was no change in pH in shrimp tissue homogenates inoculated with type E.

Shrimp tissue homogenate supported growth and toxin production by *C. botulinum* types A to E. This finding indicated that shrimp is a conducive medium for the growth of *C. botulinum* types A to E. But the low toxin titres produced by type E in

shrimp tissue homogenate indicates that it is less conducive to type E growth and toxigenesis than types A, B, C and D.

The limiting temperature for growth and toxin production by *C. botulinum* types A, B, C and D were found to be 15°C and that of type E was found to be 4°C in shrimp tissue homogenate.

The storage temperature was significant on the growth and toxin production by *C. botulinum* types A to E. Types A, B, C and D grew and produced toxin at 30° and 15°C but not at 10°C and 4°C. Rapid toxin development was noticed at 30°C by *C. botulinum* types A to E but lowering the storage temperature to 15°C progressively delayed toxin production by types A and B for 6 d, by types C and D for 8 d and by type E for 5 d. But when the packs stored at 10°C and 4°C were transferred to 30°C, growth and toxin production were noticed in the packs indicating the significance of storage temperature on growth and toxigenesis. Toxin titres developed at storage temperature 30°C were high compared to that produced at 15°C. Type E grew and produced toxin at temperatures 30°C, 15°C, 10°C and 4°C. High toxin titres were found at high storage temperature and as the storage temperature was lowered, low toxin titres were found in the shrimp tissue.

The growth and toxin production by *C. botulinum* was influenced by the growth substrate. Even within the *C. botulinum* types A to E, the growth and toxin production varied with the substrate. High toxin titres were found in types A, B, C and D inoculated packs whereas type E inoculated packs showed poor toxin production. The high toxin titres developed in types A, B, C and D inoculated packs can be attributed to the enhanced growth and toxin production by these types in shrimp tissue homogenate. Low

toxin titres in type E inoculated pack may be due to the poor growth and toxin production by type E in shrimp tissue homogenate.

Strasdine and Kelly (1967) found extracts of shrimp, oyster and crab were relatively inactive in allowing rapid germination of type E. Lerke and Farber (1971) had shown that shrimp homogenate is a suitable substrate for the growth and toxin production of *C. botulinum* types A, B and E. Studying the outgrowth and toxin production by type E in vacuum skin packaged shrimp, Garren *et al.* (1994) found toxin production by type E in shrimp. The present finding that shrimp tissue homogenate can support growth and toxin production by *C. botulinum* agrees well with the earlier reports.

Lerke and Farber (1971) reported outgrowth and toxin production by *C. botulinum* types A at 75°F (23.8°C) in shrimp tissue homogenate but storage at 10°C prevented toxigenesis by types A and B but not by E. Fletcher *et al.* (1988) reported toxigenesis by type A in scallops held at 27°C under vacuum but storage at 10°C and 4°C inhibited growth and toxin production. Similar observations were made in the present study. *Clostridium botulinum* types A, B, C and D could grow and produce toxin at 30°C and 15°C but not at 10°C. The results of the study confirmed the earlier finding of Segner *et al.* (1971b) that minimal temperature for growth of *C. botulinum* type C is very near to that accepted as limiting for growth of type A and proteolytic type B.

The minimum temperature that allowed growth and toxin production by *C. botulinum* type E in the present study was 4°C and toxin production was noticed after 35 d. Lerke and Farber (1971) could not find growth and toxin production by type E at 4°C for a period of 75 d. Patel *et al.* (1978) found toxin production by type E in marine molluscs at 4°C after 90 d. Garren *et al.* (1994) studied outgrowth and toxin production

by type E in vacuum skin packaged shrimp and found toxin production at 10°C after 6 d. But on storage at 4°C, neither growth nor toxin production was observed upto 21d. The variation noticed in the lag period at 4°C in the above studies may be attributed to the differences in the spore inoculum, and to the inhibition of growth of type E by the initial microbial flora of the tissue homogenate. The present finding also agrees well with that of Garren *et al.* (1994) in that no toxin production was noticed by type E upto 21 d at 4°C.

Table 25: Growth and toxin production in shrimp tissue homogenate inoculated with *Clostridium botulinum* and packed under vacuum

C. botulinum Strains	Inoculum level per 10g	Storage temperature (°C)	Toxin detection (d)
5A	7.2×10^5	30 15 10 4	2 6 - -
90B	1.3×10^5	30 15 10 4	2 6 - -
18C	1.6×10^6	30 15 10 4	2 8 - -
2693 D	5.0×10^5	30 15 10 4	2 8 - -
Type D 25717	1.5×10^6	30 15 10 4	2 8 - -
Type E 10660	1.0×10^3	30 15 10 4	2 5 10 35

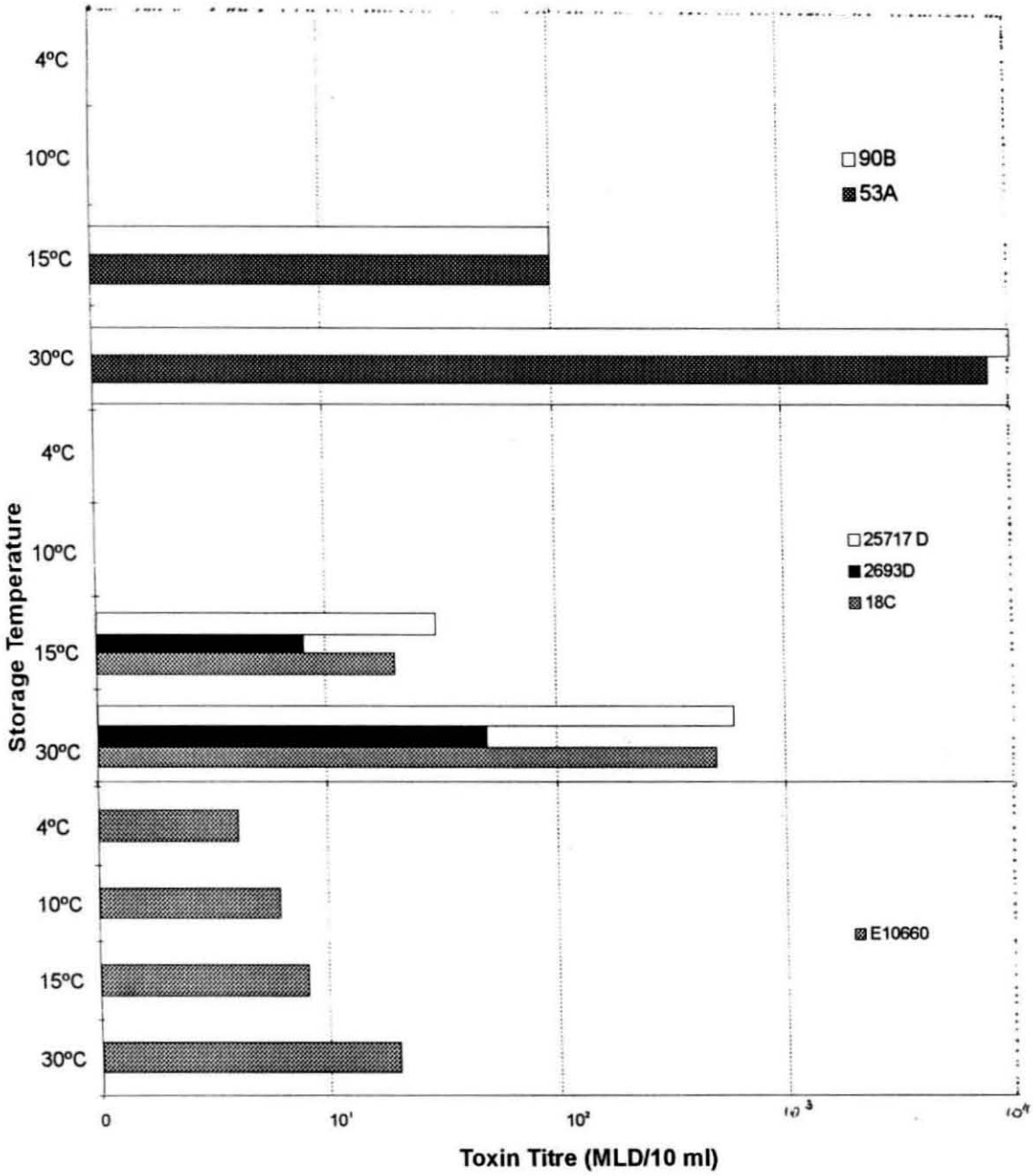

Fig. 16 Comparison of Toxin production by *Clostridium botulinum* types A to E in shrimp tissue homogenate

The significance of storage temperature on the growth and toxin production by *C. botulinum* has been reported earlier. Cann *et al.* (1965b) and Huss *et al.* (1979 a) showed that storage temperature and spore load in fish markedly influence the toxin titres in vacuum packed fish. Genigeorgis and Riemann (1979) stated that the rate of growth of *C. botulinum* type A or B may increase ten fold when the temperature increases from 15 to 25°C. Farber (1991) reported that both temperature and substrate can greatly influence the ability of non-proteolytic *C. botulinum* (types B, E, F) to produce toxin. Lindroth and Genigeorgis (1986), Garcia and Genigeorgis (1987) and Ikawa and Genigeorgis (1987) have shown that as the storage temperature is lowered the lag phase is increased. Similar results were obtained in the present study.

Most of the earlier work emphasised the significance of storage temperature on the toxin titres produced by type E in fish. The study on mullet tissue homogenates (4.6.4.1) also showed the significance of storage temperature on the toxin titres produced by *C. botulinum* types A to E. Unlike fish tissue homogenates, toxin production by type E in shrimp tissue homogenates is low. Mullet tissue was found to be superior to shrimp tissue homogenate in supporting growth and toxin production by type E. Mullet tissue offered a better balanced nutrient than shrimp on account of its high fat content for the rapid germination and multiplication of *C. botulinum* type E spores. The present finding confirms the earlier observation of Strasdine and Kelly (1967) in that extracts of cod, salmon, sole and herring enhanced germination of type E spores while extracts of shrimp, oyster and crab were relatively inactive. Other workers had also reported that fish with a high fat content provide a better medium than non fatty fish (Abrahamsson *et al.* 1965; Cann *et al.* 1965).

In recent years, there has been a renewed interest in the use of Modified Atmosphere (MA) combined with refrigeration to extend the shelf life of fresh fish at reasonable cost (Cann *et al.* 1983; Genigeorgis 1985; Gopal *et al.* 1990). But temperature abuse is common and maintenance of proper refrigeration by consumer is even worse. Unprocessed fresh and frozen shell fish have had an excellent safety record with regard to botulinal problems (Hauschild 1993). The extent to which they are processed and the conditions under which they are stored have a marked effect on the safety of the product. Therefore, strict adherence to temperature control is necessary for MAP products not only by the food producer and retailer but also by the consumer.

The naturally occurring levels of *C. botulinum* in fresh farmed shrimp was 150/100g or 15/10g. Nonproteolytic *C. botulinum* types were not encountered in shrimp in the present study (4.1). However, it is not to be assumed that they are absent in shrimp. The inoculum level of type E used in the present study was higher than the naturally occurring levels of *C. botulinum*. The present findings indicated that naturally occurring levels of *C. botulinum* in fresh shrimp will not initiate toxigenesis in shrimp stored at 4°C under vacuum for upto 35 d. At abused storage temperature of 10°C, type E or nonproteolytic *C. botulinum* types if present, could initiate growth and toxigenesis within 10 d. The study suggests that if the strict temperature control cannot be guaranteed, the storage time has to be limited. Shrimp tissue supported growth and toxin production by *C. botulinum* A to E. The study also revealed that mullet tissue was superior to shrimp tissue in supporting growth and toxin production by type E. The study also indicates that dead shrimp in shrimp farms support growth of *C. botulinum*. Hence it is recommended that proper hygiene should be maintained in shrimp farms including

frequent removal of dead shrimps to reduce the level of contamination by *C. botulinum*. The results of the study can be utilized to define control measures for predicting the safety of vacuum packaged shrimp.

4.6.5 Combination Effects

4.6.5.1 Combined effect of temperature and pH on growth and toxin production by Clostridium botulinum

The effect of pH on growth and toxin production by *C. botulinum* was studied at temperatures 30°C, 15°C, 10°C and 4°C. The minimum pH that permitted growth of *C. botulinum* type A isolate at 30°C was between 4.5 and 5.0 whereas at 15°C, it was raised to 5.0-5.5 and at 10°C and 4°C, neither growth nor toxin production was noticed over the pH range 4.5-7.0 (Table 26). The minimum pH that permitted growth of *C. botulinum* type C spores at 30°C and 15°C was 5.5 but the toxin production was delayed for a period of 10 d at 15°C. *C. botulinum* type D spores initiated growth at 30°C and pH 5.0. The toxin production was delayed for a period of 16 d. At 15°C, growth of *C. botulinum* type D was inhibited at pH 5.0. Following the transfer of tubes from 10° and 4°C to 30°C, growth and toxin production occurred in all tubes inoculated with *C. botulinum* types A and D within 12-18 d at pH 5.0 but not at pH 4.5.

Growth and toxin production by *C. botulinum* type E reference strain occurred at 30°C and 15°C over the pH range 5.0 to 5.5 whereas at 10°C, the minimum pH was between 5.5 to 6.0 and at 4°C, it was raised to 6.0 to 7. As the temperature was lowered, the minimum pH value permitting growth and toxin production by spores of *C. botulinum* was raised.

The results of the present study revealed that at suboptimal temperature, the limiting pH value permitting growth of *C. botulinum* was raised. Growth and toxin production by *C. botulinum* type A and D occurred at pH 5.0 and 30°C whereas at 15°C, neither growth nor toxin production was noticed at pH 5.0. Similarly the limiting pH value permitting growth of type E reference strain at 30°C was 5.5 whereas at 10°C, it was raised to 6.0. Similar observations were made by Segner *et al.* (1966), Lynt *et al.* (1982) and Graham and Lund (1987).

There are only limited studies on growth of individual *C. botulinum* strains (Segner *et al.* 1966; Baird-Parker and Freame 1967; Emodi and Lechowich 1969 a). Most of the investigations deal with the behaviour of mixed cultures of *C. botulinum* (Lund *et al.* 1985, 1987; Graham and Lund 1987).

In a study of single strains of type A, B and E, using an inoculum of 10^6-10^7 spores, Baird Parker and Freame (1967) found growth of type B at 30°C and pH 5.0 in 9 d whereas type A grew upto pH 5.3. In the same study, they found that at 20°C and pH 5.0, all three types failed to form vegetative cells and at pH 5.3 and 20°C, type A, B and E were able to from vegetative cells. Lund *et al.* (1985) did not find multiplication and formation of toxin by type A at 20°C and 16°C over the pH range 5.1-4.5. In a subsequent work, Graham and Lund (1987) have shown rapid germination and growth of types A and B at 30°C and pH 5.2-5.5 whereas at 20°C, growth occurred upto pH 5.4 within 26 d. They also found growth and toxin production by type B at 16°C and pH 5.5 after 14 d. The results of the present study agree well with the above observations in that type A grew at pH 5.5 after 14 d and growth and toxin production by type A did not occur

over the pH range 4.5-5.0 at 15°C whereas at 30°C rapid germination and growth of type A occurred upto pH 5.0.

The minimum pH permitting growth of type A at 30°C was 5.0 in the present study. Baird-Parker and Freame (1967) found growth and toxin production by type A at 30°C and pH 5.3 but not at pH 5.0. However, it has also been demonstrated earlier that growth and toxin production by *C. botulinum* type A occurred at pH 4.8. Several reports have demonstrated the ability of *C. botulinum* type A to grow and produce toxin at pH levels below 4.6 (Raatjes and Smelt 1979; Odlaug and Pflug 1979; Smelt *et al.* 1982; Montville 1982; Tanaka 1982; Tsang *et al.* 1985; Young-Perkins and Merson 1987; Dodds 1989). The pH tolerance of *C. botulinum* may be greatly modified by the acidulants used, concentration, type and strain of *C. botulinum* spores, sufficiently anaerobic growth medium, metabiosis and high concentrations of proteins in the medium as shown in the previous studies. The variation in the minimum pH value permitting growth of *C. botulinum* type A may be attributed to the variability in acid tolerance exhibited by different strains of *C. botulinum* and the anaerobic conditions in the growth medium.

Segner *et al.* (1966) found that minimum pH permitting growth of type E strains at 30°C was between 5.24-5.36 whereas at 8°C it was raised to 5.9-6.1. Baird-Parker and Freame (1967) have shown outgrowth of type E strain at pH 5.3 and 30°C and 20°C but not below pH 5.3. In a similar study, Emodi and Lechowich (1969a) found that the minimum pH permitting growth of six strains of type E from an inoculum of 1.5×10^6 spores were at 15.6° pH 5.4-5.6, at 10°C pH 5.6-6.0, at 5°C pH 6.2-6.4. Lund *et al.* (1990) have shown greater ability of non-proteolytic type B to grow at low pH at sub-

optimum temperature than at optimum temperature and neutral pH. Similar observations were made in the present study in that as the temperature was lowered from 15° to 10°C the minimum pH value permitting growth and toxin production by type E was raised from pH 5.5 to 6.0.

Table 26: Combined effect of pH and temperature on growth and toxin production by *Clostridium botulinum*

C. botulinum Strain	pH of the medium	No. of d required for growth and toxin production			
		30°	15°	10°	4°
53 A	7.0	1	4	-	-
	6.0	2	5	-	-
	5.5	4	14	-	-
	5.0	11	-	-	-
	4.5	-	-	-	-
18 C	7.0	1	4	-	-
	6.0	2	5	-	-
	5.5	5	10	-	-
	5.0	-	-	-	-
	4.5	-	-	-	-
2131 D	7.0	1	4	-	-
	6.0	2	6	-	-
	5.5	5	10	-	-
	5.0	16	-	-	-
	4.5	-	-	-	-
E 10660	7.0	1	3	12	28
	6.0	2	3	15	-
	5.5	3	5	-	-
	5.0	-	-	-	-
	45.	-	-	-	-

The minimum pH value permitting growth and toxin production by *C. botulinum* type C isolate at 30°C and 15°C was 5.5 but at 15°C, toxin production was delayed for a period of 10 d. The minimum pH permitting growth and toxin production by *C. botulinum* type D was raised to 5.5 from 5.0 as the temperature was lowered from 30°C to 15°C. There are no published reports of growth of *C. botulinum* type C and D at

suboptimal temperature and pH. Most of the data available are for *C. botulinum* types A and B and non-proteolytic types B and E. A decrease in pH resulted in progressively longer delays before growth of all *C. botulinum* types examined at suboptimal temperature and types C and D are no exception.

It was also observed that at pH value approaching the limit that allowed growth i.e. pH 5.0, growth and toxin production by *C. botulinum* type D was delayed for a period of 16 d at 30°C whereas at 15°C there was no growth and toxin production. This may be attributed to the fact that the proportion of spores that resulted in growth at suboptimal pH and temperature may have been low. Roberts and Thomas (1982) and Lund *et al.* (1987; 1990) had shown that the properties of spores may differ significantly between batches of the same strain and with time of storage and the probability of germination at acid pH may therefore be very variable.

The results of the present study indicate the ability of *C.* botulinum types A, C, D and E to grow in conditions that combine suboptimal temperature and suboptimal pH. However, the lag phase is increased. The study also shows that pH tolerance is a function of incubation temperature. The study suggests that an additional preservative factor should be combined with acidification and low temperature to inhibit growth of *C. botulinum* and substantially reduce the botulinum hazard associated with such fishery products.

4.6.5.2 Effect of incubation temperature on salt tolerance of *Clostridium botulinum*

The inhibition of outgrowth and toxin production by spores of *C. botulinum* types A, C, and D isolates and type E reference strain at different concentrations of sodium

chloride was studied at different incubation temperatures. The a_w values of CMM containing various concentrations of sodium chloride are shown in table 27. A delay in outgrowth time occurred with increasing salt concentrations and decreasing temperatures (table 28).

At 30°C, 10% sodium chloride prevented growth and toxin production by *C. botulinum* type A whereas 8% sodium chloride delayed growth and toxin production for a period of 13 d. But when the temperature was lowered to 15°C, 8% sodium chloride was required to prevent growth and toxin production during the 42 d of storage and 5% sodium chloride delayed growth and toxin production for a period of 18 d. Neither growth nor toxin production by type A was noticed below 15°C.

The sodium chloride level for inhibition of *C. botulinum* types C, D and E at 15°C was not much less than that at 30°C. Growth and toxin production by *C. botulinum* types C and D were observed at 15°C but not below that whereas type E grew and produced toxin at 10°C and 3% sodium chloride. The toxin production was delayed for a period of 25 d at 10°C. It has been demonstrated that at 15°C, a delay in outgrowth time of types C and D occurred at 3% and 5% sodium chloride levels and the toxin was detected after 8 and 14 d respectively.

Table 27: Influence of NaCl on the water activity a_w of CMM

Percent NaCl (w/v)	a_w
0	0.994
3	0.978
5	0.970
8	0.952
10	0.936

Table 28: Effect of Incubation temperature on salt tolerance of *Clostridium botulinum*

C. botulinum strain	Sodium chloride concentration %	No. of d required for growth and toxin production			
		30°C	15°C	10°C	4°C
53 A	0	1	3	-	-
	3	2	4	-	-
	5	6	18	-	-
	8	13	-	-	-
	10	-	-	-	-
18 C	0	1	4	-	-
	3	2	8	-	-
	5	6	12	-	-
	8	-	-	-	-
	10	-	-	-	-
2693 D	0	1	5	-	-
	3	3	8	-	-
	5	6	14	-	-
	8	-	-	-	-
	10	-	-	-	-
E 10660	0	1	2	12	28
	3	3	6	25	-
	5	-	-	-	-
	8	-	-	-	-
	10	-	-	-	-

The present study revealed that the sodium chloride concentrations permitting growth of *C. botulinum* is influenced by temperature. At 30°C and pH 7.0, growth of *C. botulinum* type A occurred upto 8% sodium chloride level whereas at 15°C, growth and toxin production was noticed only upto 5% sodium chloride. Similar observations were made by other investigators (Ohye and Christian 1967; Roberts and Smart 1976; Lynt *et al.* 1982). Salt concentrations with little inhibitory effect at optimal incubation temperature significantly delay outgrowth at lower temperatures. Studying the combined

effect of salt and temperature, Gibson *et al.* (1987) found that growth of *C. botulinum* type A in pasteurised pork slurry was delayed for a period of 60-211 d at 15°C and 4.5% sodium chloride. An extension of lag phase was noticed in the present study also at 15°C. However, the lag period was considerably shorter than that reported by Gibson *et al.* (1987). Differences in the lag period observed in the above study may be attributed to the difference in the inoculum levels or to the heat treatment given to the spores or to the difference in the rate of germination of spore crops. Lund and Wyatt (1984) have shown that the concentration of sodium chloride required to prevent growth of *C. botulinum* is influenced by the spore inoculum size. It was also demonstrated that heat activation of spores delay outgrowth and increase their sensitivity to sodium chloride (Roberts and Smart 1976; Roberts and Thomas 1982; Peck *et al.* 1995; Graham *et al.* 1996). Roberts and Thomas (1982) and Lund *et al.* (1990) have found that individual spores separated from an apparently homogeneous spore crop germinated at widely varying rates.

Growth and toxin production by *C. botulinum* type E were prevented at 5% sodium chloride and at all temperatures 4°C-30°C whereas at 3% sodium chloride growth was delayed for a period of 25 d at 10°C. It has been demonstrated in the present study that salt concentrations slightly lower than those providing inhibition tended to extend spore outgrowth time of type E reference strain at low temperature. Segner *et al.* (1966), Emodi and Lechowich (1969b) and Graham *et al.* (1996) also made similar observations on the inhibition of growth of type E. Lynt *et al.* (1982) have also reported that as the temperature of storage is lowered, less salt is required to inhibit the growth of *C. botulinum* and delay toxigenesis.

Growth and toxin production by *C. botulinum* types C and D occurred at 5% sodium chloride level and 15°C. However, a delay in outgrowth time was noticed. It has also been demonstrated that sodium chloride level required for inhibition of growth of types C and D at low temperature was not much less than that at 30°C. Most of the studies on the combined effect of temperature and sodium chloride on growth and toxin production by *C. botulinum* centered around *C. botulinum* types A, B and E. There is a lack of published data on the combined effect of temperature and sodium chloride on the inhibition of growth of *C. botulinum* type C and D.

The results of the present investigation revealed that sodium chloride concentration permitting growth of *C. botulinum* are influenced by temperature. Growth and toxin production by *C. botulinum* type A were inhibited at 15°C and 8% sodium chloride. Growth and toxin production by *C. botulinum* types C and D were not prevented at 15°C and 5% sodium chloride levels. *Clostridium botulinum* type E grew and produced toxin at 10°C and 3% sodium chloride. The study indicated that salt and temperature alone are not adequate to prevent outgrowth and toxin production by *C. botulinum*. Increasing the salt concentration of fishery product to such a level would minimise their acceptance by consumer. Therefore, the possibility of using salt concentrations considerably lower than 5% must be explored by combining temperature and sodium chloride with other factors including pH, and preservative factors such as starter cultures of lactic acid bacteria, bacteriocins or essential oils to control growth of *C. botulinum* and to maintain the bacteriological safety of the fishery products.

4.6.5.3 Inhibitory effect of combinations of pH sodium chloride and incubation temperature on growth and toxin production by *Clostridium botulinum*

The effect of incubation temperature on growth and toxin production by *C. botulinum* at pH 7.0 and 5.5 and sodium chloride concentrations of 0%, 3% and 5% is shown in table 29. At 30°C and pH 7.0 and 5.5, growth and toxin production by *C. botulinum* type A occurred at all salt levels used whereas at 15°C and pH 5.5, neither growth nor toxin production was noticed at 5% sodium chloride level.

Growth and toxin production by *C. botulinum* types C and D isolates were demonstrated at 30°C and 15°C and pH 7.0 at all salt concentrations whereas at pH 5.5 and 30°C, 5% sodium chloride prevented growth during the 42 d of storage and at 15°C and pH 5.5, growth and toxin production by types C and D were delayed for a period of 24-32 d at 3% sodium chloride level. In the case of type E strain, 5% sodium chloride was required for the complete inhibition of growth and toxin production at pH 7.0 and 5.5 and 30°. However at 15°C and 5.5, neither growth nor toxin production was noticed at 3% sodium chloride level during the 6 weeks storage.

An interaction between sodium chloride, pH and temperature was noticed in the present study. Growth of *C. botulinum* type A occurred at 15°C at pH value 7.0 and 5% sodium chloride within 9 d. As the pH value was lowered to 5.5, neither growth nor toxin production was noticed at 15°C and 5% sodium chloride. There was a delay in outgrowth even at 3% sodium chloride level for a period of 14 d. Similarly as the pH was lowered from 7.0 to 5.5, 5% sodium chloride prevented growth of *C. botulinum* types C

and D at 15°C. As the temperature and pH became limiting, less salt was required to inhibit the growth of *C. botulinum* and delay toxigenesis.

Table 29: Effect of pH, sodium chloride concentration and incubation temperature on growth of *Clostridium botulinum*

C. botulinum Inoculum	% sodium chloride	pH	No. of days required for growth and toxin production when incubated at	
			30°C	15°C
53 A	0	7.0	1	3
	3		1	4
	5		6	9
	0	5.5	4	8
	3		5	14
	5		12	-
18 C	0	7.0	1	4
	3		2	10
	5		6	18
	0	5.5	5	6
	3		19	24
	5		-	-
2693 D	0	7.0	1	5
	3		3	9
	5		8	16
	0	5.5	5	8
	3		21	32
	5		-	-
E 10660	0	7.0	1	2
	3		3	6
	5		-	-
	0	5.5	3	3
	3		12	-
	5		-	-

Several workers have assessed the combined effects of pH, temperature and water activity (a_w) on the inhibition of *C. botulinum*. The minimum a_w allowing growth of *C. botulinum* increased with decreased pH value i.e. at pH 5.5, the minimum a_w

permitting growth of *C. botulinum* type A was increased from 0.96 to 0.97 (Baird-Parker and Freame 1967). Ohye and Christian (1967) have shown that growth and survival of *C. botulinum* type B was influenced concomitantly by temperature, pH and a_w. They found that as the incubation temperature was reduced from 30°C to 20°C, the minimal a_w permitting growth increased markedly. In the same study a reduction in the pH produced a similar effect. The present findings confirm and extend those of Baird-Parker and Freame (1967) and Ohye and Christian (1967).

A similar inhibitory effect of combinations of pH, sodium chloride and incubation temperature was noticed on the growth of *C. botulinum* types C and D. As the pH was lowered from 7.0 to 5.5, 5% sodium chloride prevented growth and toxin production by types C and D. Studies on the effect of combinations of pH sodium chloride and temperature on growth and toxin production by *C. botulinum* types C and D have not been reported so far and most of the data available are from studies on *C. botulinum* types A, B and E.

At sub-optimal temperatures, the concentration of sodium chloride necessary to inhibit growth of type E *C. botulinum* was lower than that required at optimal temperature (Segner *et al.* 1966; Emodi and Lechowich 1969). Abrahamsson *et al.* (1966) and Emodi and Lechowich (1969) have shown that at 30°C, 3.5% sodium chloride permitted growth and toxin production by type E strains in 4 d, at 15.6°C it grew within 7 d, at 10°C, growth was delayed for 14 d, at 6°C it was delayed upto 120 d and at 5°C, 2.5% sodium chloride prevented growth of same strains of type E. Data presented in table 29 indicate that although 3% salt had no major effect on delay in outgrowth time of type E reference strain at 30°C and pH 7.0, at 15°C, growth was delayed for a period of 6 d.

At pH 5.5, it delayed growth for a period of 12 d at 30°C whereas at 15°C, neither growth nor toxin production were noticed during the 42 d storage. Similar observations were reported previously when unheated spores of non-proteolytic *C. botulinum* were inoculated into meat medium (Graham *et al.* 1996). Graham *et al.* (1996) found that at 4.3% sodium chloride, *C. botulinum* grew and produced toxin within 9 d at 16°C and pH 6.5 whereas at 16°C and pH 5.6, growth and toxin production were delayed for a period of 95 d. They have also shown that combinations of heat treatment and pH delayed growth and toxin production at small levels of sodium chloride at refrigerated temperature.

The present study revealed that combined stress has an immediate effect on *C. botulinum* producing a marked delay in outgrowth and toxin production. Combinations of pH 5.5 and 5% sodium chloride prevented growth of *C. botulinum* types A, C and D at 15°C whereas combinations of pH 5.5 and 3% sodium chloride prevented growth of C. botulinum type E at 15°C during the 6 weeks storage. By identifying suitable combinations of treatments, growth and toxin production by *C. botulinum* can be prevented. It has also been shown that types C and D do not pose additional problems to the food industry and measures to inactivate and control the other types of *C. botulinum* should also control types C and D. The results of the study can be applied in the formulation of foods that rely on the variables investigated to inhibit the growth of *C. botulinum* and to improve the quality and extend the shelf life.

5. SUMMARY AND CONCLUSIONS

The present investigation was carried out to understand the ecology of *C. botulinum* by focussing mainly on the distribution of *C. botulinum* in farmed fish, shellfish and fishery products and the factors controlling growth and toxin production of *C. botulinum*.

This thesis has essentially six sections

1. Distribution of *C. botulinum* in farmed and wild fish/shellfish
2. *Clostridium botulinum* in important fishery products
3. Isolation and characterisation of *C. botulinum*
4. Sensitivity of fish to *C. botulinum* toxin
5. Stability of *C. botulinum* toxin
6. Growth characteristics of *C. botulinum*

5.1 *Clostridium botulinum* in farmed and wild fish/shellfish

5.1.1 Distribution of *Clostridium botulinum* in fish/shrimp farms

The distribution of *C. botulinum* in farmed fish/shrimp and farm environment in relation to the heterotrophic bacteria was studied. Six farms were examined. The results of the present study indicate that the numbers of heterotrophic bacteria isolated from pond water mud, farmed fish and shrimp do not exceed the levels reported in wild fish from tropical waters or farmed fish from temperature waters. The contamination by *C. botulinum* was noticed in 3 out of 6 farms. The study indicates that the commensal bacterial population of fish/shrimp included *C. botulinum*. Pond water, mud and soil were found to be the source of contamination of fish/shrimp by *C. botulinum*. The incidence of *C. botulinum* was high in farmed fish/shrimp (10-20%) compared to pond

mud (11%). However, the level of contamination was higher in pond mud (11/g) than farmed fish/ shrimp. The only types of *C. botulinum* detected were types C and D. Fish viscera were seen to be a major reservoir of contamination in farmed fish. Therefore, efficient evisceration and washing of fish may reduce the level of contamination in farmed fish. Recovery of *C. botulinum* types C and D from the farms does not pose any great hazard to farm animals and humans.

The commercial pelleted shrimp feed contained high bacterial populations. The anaerobic microflora of feed is predominated by Clostridia. Clostridia also formed the predominant group among the anaerobic microflora of pond water, mud and farmed fish/shrimp. Majority of them were proteolytic indicating that they play an important role in the decomposition of organic matters in the culture ponds. They seem to be the allochthonous bacteria of culture ponds because these organisms showed remarkable changes when a large amount of organic matters were supplied during the farming operations.

Clostridium botulinum type C and D prevalent in water and mud were also encountered in farmed fish/shrimp indicating a close link in the distribution of *C. botulinum* in fish/shellfish and sediments. This finding is based on statistical analysis showing significant positive correlative between *C. botulinum* count in *Penaeus monodon* and *C. botulinum* count in the mud ($p < 0.05$).

The significant positive correlation between *C. botulinum* count of mud and the environmental factor pH indicates that this factor governs the multiplication of *C. botulinum* in mud. Ponds having earthen bottoms, excess feeds, overloading the pond with organic matter can all substantially contribute to the growth of *C. botulinum.*

Farming practices render the organically rich mud anaerobic thus allowing the multiplication of *C. botulinum*. It is not a danger but the sale of fish/shrimp contaminated with *C. botulinum* spores is likely to increase. Fish botulism is associated with *C. botulinum* type E only. Therefore recovery of *C. botulinum* types C and D from the farms examined do not pose any great hazard to farm animals.

The present study indicates that the level of contamination of farmed fish/shrimp and pond mud by *C. botulinum* and heterotrophic bacteria do not exceed those reported for farmed and wild fish from temperate waters. Therefore, farm raised fish/shrimp do not pose a great hazard to human health than do wild fish. Since aquacultured fish/shellfish can be brought to the market faster than wild fish, they probably pose a considerably lower microbiological risk than wild fish. The level of contamination of pond mud by *C. botulinum* is high compared to farmed fish/shrimp. As the farm environment is contaminated by *C. botulinum*, it is unlikely that contamination will ever be totally eliminated from farmed fish/shrimp. However, clean healthy farm management in aquaculture can substantially reduce the contamination. By periodical cleaning of the pond bottom, the contamination can be kept at low level. The top layer of bottom sediments of the ponds should be removed and the pond bottom must be treated with quick lime after each farming operations to prevent any build up in the number of *C. botulinum* present in the pond bottom. It is also suggested that dead fish should be removed from the farms as soon as they are found, appropriate feeding strategy should be adopted to regulate excess feed to reduce the organic load of the farm and the dissolved oxygen level should be maintained at the optimum level by aeration/exchange of water to prevent the multiplication of *C. botulinum* in the culture system. In farmed fish/shrimp,

the risk of botulism can be increased by modern fish preservation process such as Modified Atmosphere Packaging, minimally processed foods etc. as they foster the growth of *C. botulinum* if processing is not proper. The recovery of *C. botulinum* from farmed fish/shellfish emphasises the need for promoting good sanitation and appropriate methods of preservation to ward off post process contamination. Proper handling and quality control immediately after harvest should result in high quality and safe products from farm raised fish and shellfish.

5.1.2 Distribution of *Clostridium botulinum* in coastal waters

The distribution of *C. botulinum* in mud and bivalve samples from the west coast and east coast was examined. The results of the survey indicated that the aquatic environment is contaminated predominantly with *C. botulinum* types C and D followed by type A. The incidence of *C. botulinum* was higher in the west coast mud (33%) compared to East coast mud (25%). The overall contamination level in bivalves was 24%.

The incidence of *C. botulinum* in the aquatic environment of India is found to be lower than that of temperate water but considerably higher than that of other tropical waters. The predominance of *C. botulinum* types C and D in the aquatic environment of India is not unexpected as surveys of tropical waters indicate predominance of types C and D. The distribution of *C. botulinum* in shellfish is closely related to their occurrence in sediments.

The incidence of *C. botulinum* in farmed and wild bivalves indicates the contamination of the water bodies used for aquafarming. The level of contamination of farmed and wild bivalves is very low. Therefore, it will not be a danger as such.

Psychrotrophic strains of *C. botulinum* are noticeably absent in bivalves. The presence of *C. botulinum* type A in shellfish may have public health implications. Isolation of *C. botulinum* from shellfish indicates that they can be a potential hazard to human health. Mishandling of shellfish during transportation and retailing may influence the population of *C. botulinum* and affect its safety. Unlike many other foods shellfish are usually eaten in many countries either raw or lightly cooked often with digestive tracts. This is a risk factor.

With the expansion of the marine farming of fish/shellfish in the last few years, more and more marine species are being farmed in the coastal waters. The presence of *C. botulinum* in sea water makes the contamination a constant threat. The present investigation concludes that there is considerable contamination with *C. botulinum* in the marine and brackish water environments. In view of the prevalence of *C. botulinum,* it is of considerable importance to adequately remove these pathogens from shellfish either by self purification in clean sea water or by adequate processing to make it fit for human consumption.

5.1.3 Investigations on the incidence of *Clostridium botulinum* in cultivable fish/ shellfish and trash fish

Fish and shellfish samples caught from the wild, which are used in aquaculture were examined for *C. botulinum*. Of the 20 samples of fish, 20 percent harboured *C. botulinum*. The frequency of contamination on the surface of fish was 20 percent while that in intestine was 15 percent. Types A and D predominated in fish followed by type C. In shellfish incidence of *C. botulinum* was 25 percent (6/24) with type C predominating followed by type D and A. The incidence of *C. botulinum* in shellfish

was higher than fish. The overall contamination of fish and shellfish was 23% with types C and D predominating followed by type A. In wild freshwater fish and shellfish examined incidence of *C. botulinum* was 20% (2/10). *Clostridium botulinum* was detected only in freshwater prawn *Macrobrachium rosenbergii*. Twenty seven percent (8/30) of the trash fish samples showed contamination predominantly by *C. botulinum* types A, C and D followed by type B. The frequency of contamination of fish on the surface was 25 percent while that of intestine was 5 percent. The overall contamination of cultivable fish/shellfish and trash fish by *C. botulinum* was 24 percent with type C predominating followed by types D, A and B. Psychrotrophic *C. botulinum* strains were noticeably absent.

The investigations on the incidence of *C. botulinum* in cultivable fish/shellfish and trash fish reveal that marine, brackishwater and freshwater environments in India are contaminated with *C. botulinum*. *Clostridium botulinum* types C and D are the predominant types in all these environments. *Clostridium botulinum* has a widespread spectrum of distribution as reflected by its isolation from different sources. The present study indicates that the overall contamination level of fish/shellfish (24%) does not exceed that reported for wild fish from temperate waters and is also within the range reported for tropical waters. The study also indicates that cultivable fish/shellfish and trash fish do not pose a great hazard to human health than do wild fish from temperate water because psychrotrophic strains of *C. botulinum* are noticeably absent. Most samples harboured type C (9%), types A and D (8% each) and type B (1%).

The frequency of contamination of fish was more on the surface than the intestine. The present finding confirmed the earlier finding that *C. botulinum* contamination on the

surface is most common in pelagic fish and contamination of the gut is most common in bottom feeding fish.

The results of the study indicate that contamination of the culture system is also possible with *C. botulinum* types associated with wild fish/shellfish through water, seed from natural sources or trash fish feed used for aquafarming. As aquaculture expands throughout the world, there is the possibility of transfer of live fish and fish eggs from country to country. With such transfers, there is possibility of introduction of psychrotrophic *C. botulinum* types from exotic fish, fish seed and it will be a problem for aquaculture. This necessitates adoption of appropriate quarantine measures at destination. The presence of *C. botulinum* types A and B, causative agents of human botulism, in fish/shellfish may have both ecological and public health implications. Therefore, fish/shellfish should be properly handled to reduce the contamination in culture systems and also to avoid public health hazard.

The recent increase in aquaculture of fish/shellfish in coastal waters makes it important to know the ecology of *C. botulinum* in various environments. It is still debated as to whether *C. botulinum* types C and D can cause human botulism. However, the potential hazards of type C and D human botulism is clearly indicated. The occurrence of *C. botulinum* in fish/shellfish poses a risk to public health. Trash fish if used as feed in the culture of carnivorous fishes should be properly handled and adequately processed to destroy the organism or toxin to keep the contamination at low level and to prevent infection of farm animals. The present study also suggests that the culture system should be monitored regularly for the presence of psychrotrophic

C. botulinum strains as there are chances of introduction of these strains from contaminated fish/fish seed.

5.2 *Clostridium botulinum* in processed fishery products

5.2.1 Incidence of *Clostridium botulinum* in chilled stored farmed shrimp

The results of the chilled storage study of farm raised shrimp revealed contamination of shrimp by *C. botulinum* type D and that spores of *C. botulinum* survived chilling temperature. Neither the growth nor the toxin production was noticed in shrimp during chilled storage. The level of contamination by *C. botulinum* was low. Nonproteolytic *C. botulinum* types were not encountered in chilled stored shrimp. The toxin production by *C. botulinum* was noticed in packed shrimp meat stored at 30°C under vacuum. This indicates that shrimp meat is a conducive medium for growth and toxin production by *C. botulinum* type D. The present study points out that *C. botulinum* is a potential hazard in chilled vacuum packed foods which usually have minimal heat processing. The study also indicates that *C. botulinum* present in shrimp farms can multiply in dead shrimp and contaminate more number of shrimp in the culture system. High standards of hygiene in the shrimp farms and processing facilities are essential to keep the contamination at low level in order to ensure safety of aquacultured products.

5.2.2 Incidence of *Clostridiudm botulinum* in frozen fishery products

The incidence of *C. botulinum* in frozen fishery products was examined. Of the 41 samples analysed, 15% were found to be contaminated with *C. botulinum* types A, B, C and D. Frozen farm raised shrimp and mussel showed contamination by *C. botulinum*

at the level of 40 per 100g. Psychrotrophic *C. botulinum* strains were not detected in frozen fishery products.

The present study indicates that frozen fishery products are contaminated with *C. botulinum* only to a lesser extent and *C. botulinum* spores are highly resistant to freezing. *Clostridium botulinum* types A and B, associated with frozen fish/shellfish, are hazardous to humans. There is a possibility of introduction of these types into fish farms through feed compounded with contaminated trash fish.

There are numerous reasons to estimate the size and composition of *C. botulinum* population in frozen foods. Freezing certainly stops the growth of *C. botulinum* during storage. Some frozen foods however are designed to be eaten without further cooking and others may not necessarily be heated to pasteurising temperature by all consumers. Such food can turn to be a risk factor. Psychrotrophic *C. botulinum* strains if present can multiply at 3.3°C. If temperature abuse occurs, even proteolytic strains multiply and produce toxins. The present study suggests that for prolonged frozen storage of fish/shellfish, strict temperature control is necessary to prevent multiplication of C. botulinum and to ensure quality and safety of the product.

5.2.3 Distribution of *Clostridium botulinum* in cured fishery products

The distribution of *C. botulinum* in cured fishery products was studied. A total of 40 cured fish/shellfish samples procured from local retail markets were examined and *C. botulinum* was detected in 13% of the samples with type D predominating followed by type C. Wide variations in salt concentration and moisture level were observed in cured fish samples.

The present study indicates a low incidence of *C. botulinum* and predominance of

types C and D in cured fishery products. *Clostridium botulinum* spores remain viable at water activity (a_w) level 0.75 and are able to grow and produce toxin after rehydration of such foods. The detection of *C. botulinum* in cured fish indicates either post processing contamination or the ability of these *C. botulinum* strains to survive the salting process during the manufacture of cured fish. Wide variations in the salt concentration of cured fishery products indicate inadequate control of the brining process. The incidence of *C. botulinum* in cured fishery products should emphasise the need for adequate brining and drying to protect these products until their final use. Proper hygienic conditions in the curing yards are to be encouraged for the production of good quality products.

The results of the study reveal that it is not possible to completely eliminate *C. botulinum* from fish and fishery products. The ubiquitous distribution of *C. botulinum* in nature complicates its elimination from the processing environment. However, it is possible to introduce some measures at different stages in the long chain of events from production to consumer to keep the contamination at a low level and to prevent the multiplication and toxin production by *C. botulinum*. Chilling, freezing and curing processes prevent multiplication and toxin production by *C. botulinum*. The recovery of *C. botulinum* types A and B, the causative agents of human botulism from frozen fish is a matter of concern. Though *C. botulinum* types C and D are not considered as hazardous to humans, the potential of type C and D human botulism exists. Therefore it is very important to keep the fresh fish/shellfish chilled to near zero (ice) at all times before processing. Cross contamination from raw material to final product must be avoided by maintaining good hygiene. The ecology of *C. botulinum* in foods is such

that, if proper hygiene and cooking practices are followed, the likelihood of human intoxications is much reduced.

5.3 Isolation and characterisation of *Clostridium botulinum*

Isolation and characterisation of *C. botulinum* encountered in fish and fishery products were done. Isolation of *C. botulinum* types A-D from the enrichment cultures was obtained by a preheating treatment of the culture at 80°C from 10 min and streaking on a solid medium (TSGYA). Isolation of *C. botulinum* type E reference strain was achieved by alcohol treatment. The strains isolated were identified by toxin neutralisation tests in mice. Morphologically all *C. botulinum* types A-E examined were similar but culturally non-proteolytic type E, mildly proteolytic types C and D and proteolytic types A and B differed considerably. Some strains of *C. botulinum* types C and D lost toxigenicity upon isolation and maintenance in laboratory media indicating the involvement of bacteriophages in toxin production.

Because *C. botulinum* toxins are serologically highly specific, it is seldom necessary to isolate the organism in survey work to study the incidence of the organism. In order to understand the properties of *C. botulinum* types and the factors affecting their growth and toxin production in fishery products, their successful isolation and identification are highly essential. Such investigations do have tremendous practical application in fixing risk factors for food safety and protecting human health.

5.4 Sensitivity of fish to *Clostridium botulinum* toxin

Sensitivity of fish to *C. botulinum* types A-E toxins were investigated. All five toxin types A to E were toxic to *Oreochromis mossambicus*. For toxin types A to D,

O. mossambicus were considerably more resistant than mice and for type E toxin, fish was more sensitive. The minimum Intrapetrioneal (IP) dose of type E toxin for fish was one half of the minimum lethal dose for mice.

The present study indicates that fish is susceptible to *C. botulinum* toxins and fish can be a good alternative to mice as laboratory animals for toxicity studies of type E *C. botulinum*. Even though fish are carriers of *C. botulinum* in aquaculture farms, they are susceptible to its preformed toxin. The study also points out that under favourable environment, *C. botulinum* types prevalent in fish farms can multiply and cause infections in the farmed species. Therefore it is suggested that good hygiene should be maintained in fish/shrimp farms to keep the contamination at low level.

5.5 Stability of *Clostridium botulinum* toxins

The effect of temperature on the stability of *C. botulinum* types A to E toxins were investigated. The botulinum toxin from *C. botulinum* types A to E were stable at -15°C for a period of 180 days. Standing at room temperature at pH 7.0 resulted in a gradual loss of toxicity of type D toxin. Prolonged incubation of *C. botulinum* types A and D toxins at 37°C and 40°C also resulted in a gradual loss of toxicity. Thermal resistance of type D toxin is slightly higher than that of type A in fish muscle medium. Heating at 80°C for 10 min inactivated the toxin of types A and D to no detectable toxin.

The results of the study reveal that heating is the only practical means of inactivating botulinum toxins in foods. Freezing or subsequent frozen storage and thawing caused no destruction of botulinum toxins of types A to E. The normal cooking procedure is sufficient to inactivate botulinum toxin. The study points out that measures to control type A toxin should also control type D toxin in fishery products.

5.6 Growth characteristics of *Clostridium botulinum*

The influence of temperature on growth and toxin production by *C. botulinum* types A to E was studied. The limiting temperature permitting growth and toxin production by *C. botulinum* types A to D was 15°C and that of type E was 4°C. A delay in outgrowth time occurred with the lowering of incubation temperature. The present study has shown that the minimal temperature for growth of types C and D is very near to the limiting temperature for growth of types A and B and hence, types C and D appears to pose no additional problems to the fish processing industry.

The present study indicates that only slow growth occur when fishery products contaminated with types A to D are kept at mild abusive temperature (15°C) and no growth at chilled storage. Only *C. botulinum* type E initiates growth and toxin production at chilled storage (4°C). It is therefore, necessary to include other safety factors in processed fishery products in addition to temperature to ensure that *C. botulinum* is inhibited and toxin formation cannot occur.

Effects of temperature and substrate on toxin production by C. botulinum type B were studied. The optimum temperature for toxin production was found to be 37°C with maximum toxin concentrations being attained in 72h. Maximum growth and toxin production were noticed in cooked meat medium at 37°C. The present investigation revealed that temperature and substrate influence toxin production by *C. botulinum* type B.

Studies on the influence of pH on growth and toxin production by *C. botulinum* type A to E have shown the limiting pH permitting growth and toxin production by *C. botulinum* type A to be 4.8, type B to be 5.0, type C and D to be 5.0-5.2 and type E to

be 5.4. Minor variations in acid tolerance were noticed among strains of *C. botulinum* types C and D. Inhibition of growth of *C. botulinum* increased as the pH decreased from 7.0 to 4.6. The study shows that growth and toxin production by *C. botulinum* types A and B are not prevented at acidic pH. Therefore, it is suggested that other safety factor should be included in addition to acidification to prevent growth and toxin production by *C. botulinum* in processed fishery products. An understanding of the mechanism by which an acidulent exerts its inhibitory effect either alone or in combination on *C. botulinum* is of great importance in establishing hazards associated with consumption of acid foods.

The minimum water activities (a_w) permitting growth and toxin production by *C. botulinum* types A to E were investigated in sodium chloride containing media and were found to be 0.952, 0.97 and 0.976 respectively for types A and B spores, types C and D spores and type E spores. These values corresponded to sodium chloride concentrations of 8%, 5% and 3% respectively. The study indicates that salt exerts antibotulinal effects resulting in inhibition of growth and toxin production by *C. botulinum* spores. *Clostridium botulinum* type E possesses a level of salt tolerance considerably below that recognised for types A and B. The study points out that 10% sodium chloride is adequate for complete botulism control and increasing the salt concentration of fishery product to such a level would minimise their acceptance by consumer. Therefore the possibility of using salt concentrations considerably lower than 10% must be explored by combining other factors such as pH temperature etc. to exhibit sufficient inhibitory activity against botulinal growth.

The effect of vacuum packaging of fish/shrimp on growth and toxin production by *C. botulinum* types A to E was studied in mullet and shrimp tissue homogenates. *Clostridium botulinum* types A to D grew and produced toxin in mullet and shrimp tissue homogenates at 30°C and 15°C while type E grew over a temperature range of 4°C to 30°C. At 4°C, type E grew and produced toxin on 28th day in mullet tissue and on 35th day in shrimp tissue homogenates. The present study indicates that vacuum packaging in combination with low temperature storage may not provide the safety required for extended storage of fresh fish products with respect to outgrowth and toxin production by type E/non-proteolytic strains of *C. botulinum*. Non-proteolytic *C. botulinum* strains are noticeably absent in fish and shellfish from coastal waters of India. The study points out that if non-proteolytic *C. botulinum* spores are present in products, even proper refrigeration may not be sufficient as these strains may grow at 4°C. Strict adherence to temperature control is necessary for MAP products not only by the food producer and retailer but also by the consumer. The naturally occurring levels of *C. botulinum* in fish/shellfish are found to be lower than the inoculum level used in the present study. The study indicates that the naturally occurring levels of *C. botulinum* in fish/shellfish will not initiate toxigenesis in vacuum packed fish stored at 4°C for upto 21-25 d and in vacuum packed shrimp stored at 4°C for upto 35 d. If the strict temperature control cannot be guaranteed, the storage time has to be limited. The results of this investigation can be utilised to define control measure for predicting the safety of vacuum packaged fish/shellfish.

The present study points out the significance of storage temperature on growth and toxin production by *C. botulinum* in fish/shrimp under vacuum pack. Mullet and

shrimp tissues are conducive medium for the growth and toxin production by *C. botulinum* types A to E. Mullet tissue is superior to shrimp tissue in supporting growth and toxin production by type E. The study indicates that in fish/shrimp farms, dead fish provide an excellent substrate to support growth of *C. botulinum*. Therefore, it has to be removed as soon as it is found to keep the contamination at low level. As mullet and shrimp tissues are good substrates for growth and toxin production by *C. botulinum*, a potential hazard does exist, if products are stored above 4°C in Modified Atmospheres/ vacuum pack. The extent to which fresh/frozen fish is processed to increase shelf life or to prepare new products and the conditions under which they are stored, therefore, have a marked effect on the potential problems from *C. botulinum*. Successful control *of C. botulinum* in vacuum packed/ Modified Atmosphere Packaged fishery products may lie in a better understanding of the combined effects of well chosen environmental variables and preservative factors.

The effect of incubation temperature on acid tolerance of *C. botulinum* was also studied. At sub-optimal temperature, the limiting pH value permitting growth and toxin production by *C. botulinum* was raised. The results of the study indicate a greater ability to grow in conditions that combine sub-optimal temperature and sub-optimal pH, of C. botulinum types A, C, D and E. However, the lag phase is increased. The study suggests that an additional factor should be combined with acidification and low temperature to inhibit growth of *C. botulinum* and substantially reduce the botulinum hazard associated with such fishery products.

The effect of incubation temperature on salt tolerance of *C. botulinum* was also investigated. A delay in outgrowth time occurred with increasing salt concentrations and

decreasing temperatures. The results of the present investigation revealed that sodium chloride concentrations permitting growth of *C. botulinum* are influenced by temperature. Growth and toxin production by *C. botulinum* type A was inhibited at 15°C and 8% sodium chloride. *Clostridium botulinum* type E grew and produced toxin at 10°C and 3% sodium chloride. Growth and toxin production by *C. botulinum* types C and D was not prevented at 15°C and 5% sodium chloride levels. The present study points out that salt and temperature alone are not adequate to prevent outgrowth and toxin production by C. *botulinum.* By combining temperature and sodium chloride, it has been shown that 5% sodium chloride is required for complete control of *C. botulinum* at 10°C. Increasing the salt concentration of fishery product to such a level would minimise their acceptance by consumer. Therefore, the possibility of using salt concentrations considerably lower than 5% must be explored by combining temperature and sodium chloride with other factors such as pH and preservative factors including starter cultures of lactic acid bacteria, bacteriocins or essential oils to control growth of *C. botulinum* and to maintain the bacteriological safety of the fishery products.

The inhibitory effects of combinations of pH, sodium chloride and incubation temperature on growth and toxin production were evaluated. Growth of *C. botulinum* type A occurred at 15°C and pH value 7.0 and 5% sodium chloride within 9 d. As the pH value was lowered to 5.5, neither the growth nor the toxin production was noticed at 15°C and 5% sodium chloride. Combinations of pH 5.5 and 5% sodium chloride prevented growth of *C. botulinum* types A, C and D at 15°C whereas combinations of pH 5.5 and 3% sodium chloride prevented growth of *C. botulinum* type E at 15°C during the 6 weeks storage. The present study indicates that combined stress has an immediate effect

on *C. botulinum* producing a marked delay in outgrowth and toxin production by types A, C, D and E. By identifying suitable combinations of treatments, growth and toxin production can be prevented. It has also been shown that types C and D do not pose additional problems to the food industry and measures to inactivate and control the other types of *C. botulinum* should also control types C and D. The results of the study can be applied in the formulation of foods that rely on the variables investigated to inhibit the growth of *C. botulinum* and to improve the quality and extend the shelf life.

The present study is a valuable contribution to the field of ecology, food hygiene and food processing. The data are important to environmental and food hygiene, veterinary medicine and medicine. The study not only provides data on the ecological behaviour of *C. botulinum* and its aquatic environmental reservoir in India, but also gives a valuable and important insight into

1) the factors controlling *C. botulinum* growth and toxigenesis

2) interaction of intrinsic and extrinsic factors to define the combination of factors where growth of *C. botulinum* cannot possibly occur, even after prolonged incubation.

Changes in the microbial ecology may influence the ultimate safety of the products. In the present study, data on growth and survival were considered not only from an ecological point of view but also from the stand point of the epidemiologist or hygienist. It is unlikely that contamination by *C. botulinum* will ever be totally eliminated from fish/shellfish. However, a thorough understanding of the properties of this group of organisms and their toxins will help to minimise the risk, to avoid the

development of new products or processes which may provide an opportunity for low numbers of *C. botulinum* spores to grow and produce toxin.

The present investigation gives sufficient indication of the future needs in research on the ecology of *C. botulinum*. Psychrotrophic *C. botulinum* strains are noticeably absent in the present study. As aquaculture expands, there is greater transfer of live fish and eggs from country to country which may lead to introduction of the above strains of *C. botulinum* and in order to ensure product safety, the occurrence of *C. botulinum* should be monitored regularly in culture systems. The detection of *C. botulinum* still depends finally on the identification of one or other of the toxins by mouse bioassay. In recent years increasing emphasis has been placed on the need to reduce the time involved and to introduce automated systems into routine microbiology. Therefore, the possible use of alternative methods such as Enzyme Linked Immuno Sorbent Assay (ELISA), polymerase chain reaction (PCR), or other nucleic acid based techniques may be explored to develop rapid diagnostic methods for the detection of *C. botulinum* types A to F. It is of great importance for epidemiological and environmental studies.

The strains of *C. botulinum* types C and D lose toxigenicity upon isolation and maintenance in laboratory media. Although recent work on bacteriophage has explained to some extent the loss of toxicity of *C. botulinum* types C and D, much remains to be understood concerning toxin production. Further research is needed to determine the role of phages and plasmids in the growth and toxin production by *C. botulinum* and transfer of toxicity to non-toxigenic clostridia by botulinum toxin gene through vectors which

may provide valuable information in future genetic and epidemiological studies of *C. botulinum*.

Much remains to be done on the inhibitory effect of combinations of factors i.e. combinations of intrinsic, extrinsic and preservative factors on the growth and toxin production by *C. botulinum*. Recent trends are towards fewer additives and use of natural and consumer acceptable preservation methods. Modified Atmosphere Packaging (MAP) and 'Sousvide' processing are the most promising technologies in modern food preservation methods. More information is needed on the behaviour of *C. botulinum* in fishery products especially with regard to the use of different packaging materials, manipulation of the gaseous environment and combined use of intrinsic, extrinsic and preservative factors. The inhibitory effect of additional barriers such as addition of starter cultures of lactic acid bacteria, bacteriocins, enzymes, essential oils or use of GRAS-Compounds may also be investigated to provide safety required for extended storage of fishery products. The present study on interaction of factors have been done using culture media and only one inoculum level. The interaction of factors using fish/shellfish substrates as well as commercially processed products have to be investigated using different inoculum levels to quantify the probability of growth of *C. botulinum* in a model food system and to predict the shelf life of the product. Such experimental work has tremendous practical importance. It also gives the customer and industry the real benefits. With ever increasing consumption of processed fishery products, driven by changing life styles, the emphasis on high quality food additives may perhaps offer solutions to the challenges of the fish processing industry in the 21st century.

6. REFERENCES

Abeyta, C. Jr. (1983) Bacteriological Quality of fresh seafood products from Seattle retail markets. *J. Food Protect.* 46(10), 901-909.

Abrahamsson, K. (1967) Occurrence of types E *Clostridium botulinum* in smoked eel. In *"Botulism 1966"*. (Eds. Ingram, M. and Roberts, T.A.) p 73-75. London, Chapman and Hall Ltd.

Abrahamsson, K., De Silva, N. and Molin, N. (1965) Toxin production by *Clostridium botulinum* type E in vacuum-packaged irradiated fresh fish in relation to changes of the associated microflora. *Can. J. Microbiol.* 11, 523-529.

Abrahamsson, K., Guttmer, B. and Molin, N. (1966). The effect of temperature on toxin formation and toxin stability of *Clostridium botulinum* type E in different environments. *Can. J. Microbiol.* 12: 385-394.

Acuff, G., Izat, A.L. and Finne, G. (1984). Microbial flora of pond reared Tilapia (*Tilapia aurea*) held on ice. *J. Food Protect.* 47 (10), 778-780.

Ala-Huikku, K., Nurmi, E., Pajulahti, H. and Raevuori, N. (1977) The occurrence of *Clostridium botulinum* type E in finnish trout farms and the prevention of toxin formation in fresh salted vacuum-packed trout fillets. *Nordisk Veterinaer medicin.* 29, 386-391.

AOAC (1990) *Official Methods of Analysis* (Ed. Helrich, K.) 15th edition. Arlington, Virginia, U.S.A., AOAC Inc.

Austin, B. and Austin, D.A. (1993) *Bacterial fish pathogens*: Disease in farmed and wild fish. Second edition pp. 376. London, Ellis Horwood.

Azuma, R. and Itoh, T. (1987) Botulism in water fowl and distribution of *Clostridium botulinum* type C in Japan. In *"Avian Botulism - An International Perspective"*. (Eds. Eklund, M.W. and Dowell, V.R. Jr.) pp 167-187. Springfield, IL, USA, Charles C. Thomas.

Bach, R., Wenzel, S., Müller Prasuhn, G. and Gläsker, M. (1971) Farmed trout as a carrier of *Clostridium botulinum* and a cause of botulism. III Evidence of *Clostridium botulinum* type E on a fish farm with processing station and in fresh and smoked trout from different sources. *Archiv. fur Lebensmittel hyg.* 22, 107-112.

Baird-Parker, A.C. and Freame, B. (1967) Combined effect of water activity, pH and temperature on the growth of *Clostridium botulinum* from spore and vegetative cell inocula. *J. Appl. Bacteriol.* 30, 420-429.

Baker D.A. and Genigeorgis, C (1990) Predicting the safe storage of fresh fish under modified atmospheres with respect to Clostridium botulinum toxigenesis by modelling length of the lag phase of growth. *J. Food Protect.* 53(2), 131-140.

Baker, D.A., Genigeorgis, C. and Garcia, G. (1990) Prevalence of *Clostridium botulinum* in seafood and significance of multiple incubation temperatures for determination of its presence and type in fresh retail fish. *J. Food Protect.* 53(8), 668-673.

Barnes, E.M. (1985) Isolation methods for anaerobes in foods. *Int. J. Food Microbiol.* 2, 81-87.

Barnes, E.M. (1986) Anaerobic bacteria of the normal intestinal microflora of animals. In *"Anaerobic bacteria in habitats other than man"* (Eds. Barnes, E.M. and Mead, G.C.) pp.225-238. Oxford, London, Blackwell Scientific Publications.

Barnes, E.M. and Mead, G.C. (1986) *Anaerobic bacteria in habitats other than man.* Oxford, London, Blackwell Scientific Publications.

Bartl, V. (1973) Semipreserved foods and food poisoning. In *"The Microbiological Safety of Food"* (Eds. Hobbs, B.C. and Christian, J.H.B.) pp 89-106. London, Academic Press.

Baumgart, J. (1972) Occurrence and demonstration of *Clostridium botulinum* type E in salt water fish. *Archiv. fur Lebensmittel hyg.* 23, 34-39.

Beirne, D.O. (1990) Chilling combined with modified atmosphere packaging. In *"Processing and Quality of foods"*. Vol. 3. *Chilled foods. The Revolution in Freshness* (Eds. Zenthen, P., Cheftel, J.C. Eriksson, C., Gormley, T.R., Linko, P. and Paulus, K.) pp. 3.190-3.203. London, Elsevier Applied Science.

Bonventre, P. and Kempe, L.L. (1960). Physiology of toxin production by *Clostridium botulinum* types A and B. *J. Bacteriol.* 79(1), 18-23.

Bott, T.L., Deffner, J.S., McCoy, E. and Foster, E.M. (1966) *Clostridium botulinum* type E in fish from the Great Lakes. *J. Bacteriol.* 91, 919-924.

Bott, T.L., Jodie Jr. J., Foster, E.M. and Sugiyama, H. (1968) Possible origin of the high incidence of *Clostridium botulinum* type E in an inland Bay (Green Bay of Lake Michigan) *J. Bacteriol.* 95(5), 1542-1547.

Boyd, J.W. and Southcott, B.A. (1968) Evaluation of fish homogenates for use as substrates in *Clostridium botulinum* type E investigations. *J. Fish. Res. Bd. Canada.* 25(8), 1745-1747.

Boyd, J.W. and Southcott, B.A. (1971). Effects of sodium chloride on outgrowth and

toxin production of *Clostridium botulinum* type E in cod homogenates. *J. Fish. Res. Bd. of Canada*. 28, 1071-1075.

Bradshaw, J.G., Peeler, J.T. and Twedt, R.M. (1979). Thermal inactivation of *Clostridium botulinum* toxins types A and B in buffer and beef and mushroom patties. *J. Food Sci*. 44, 1653-1657.

Bulatova, T.I., Chulkova, I.F., Anishimova, L.I. and Kazdobina, I.S. (1973). Detection in the U.S.S.R. of *Clostridium botulinum* type F and its differential diagnosis with type E. *Zhur. Mikrobiol. Epidemiol. Immunobiol*. 10, 105-107.

Burns, G.F. and Williams, H. (1975) *Clostridium botulinum* in Scottish fish farms and farmed trout. *J. Hyg. Camb*. 74, 1-6.

Cann, D.C. (1977) Bacteriology of shellfish with reference to international trade. *Proc. Conf. Handl. Process. Mark. Tropical fish*. 377-394.

Cann, D.C. (1984) Developments in the modified atmosphere packaging of fish. *IFST Proc.*, 17(1), 15-16.

Cann, D.C. and Taylor, L.Y. (1982) An outbreak of botulism in rainbow trout *Salmo gairdneri* Richardson farmed in Great Britain. *J. Fish Dis*. 5, 393-399.

Cann, D.C. and Taylor, L.Y. (1984) An evaluation of residual contamination by *Clostridium botulinum* in a trout farm following an outbreak of botulism in the fish stock. *J. Fish Dis*. 7, 391-396.

Cann, D.C., B.B.Wilson and Hobbs, G. (1968) Incidence of *Clostridium botulinum* in bottom deposits in British coastal waters. *J. Appl. Bacteriol*. 31, 511-514.

Cann, D.C., Hobbs, G. and Wilson, B.B. (1968) The incidence of *Clostridium botulinum* in bottom deposits in British coastal waters. *J. Appl. Bacteriol.* 31, 511-514.

Cann, D.C., Houston, N.C., Taylor, L.Y., Smith, G.L., Thomson, A.B. and Craig, A. (1984). *Studies of salmonids packed and stored under a modified atmosphere.* Torry Research Station, Aberdeen. 53, pp.

Cann, D.C., Shewan, J.M., Wilson, B.B., Roberts, T.A. and Rhodes, D.N. (1966) A comparison of toxin produced by *Clostridium botulinum* type E in irradiated and unirradiated vacuum-packed fish. *J. Appl. Bacteriol.* 29, 540-548.

Cann, D.C., Smith, G.L. and Houston, N.C. (1983). Further studies on marine fish stored under modified atmosphere packaging. pp 61. *Technical report*, Torry Research Station, Aberdeen, U.K.

Cann, D.C., Taylor, L.Y. and Collett, J.M. (1980) Botulism and fishery products: evaluation of the role of vacuum packaging. *1st World Cong. Food borne Inf. Intox.*, Robert von Ostertag Institute, Berlin, pp 826-833.

Cann, D.C., Taylor, L.Y. and Hobbs, G. (1975) The incidence of *Clostridium botulinum* in farmed trout raised in Great Britain. *J. Appl. Bacteriol.* 39, 331-336.

Cann, D.C., Wilson, B.B., Hobbs, G. and Shewan, J.M. (1965b) The growth and toxin production of *Clostridium botulinum* type E in certain vacuum packed fish. *J. Appl. Bacteriol.* 28(3), 431-436.

Cann, D.C., Wilson, B.B., Hobbs, G. and Shewan, J.M. (1967) *Clostridium botulinum* type E in the marine environment in Great Britain. *In "Botulism 1966"* (Eds. Ingram, M. and Roberts, T.A.) pp 62-65. London, Chapman and Hall.

Cann, D.C., Wilson, B.B., Hobbs, G., Shewan, J.M. and Johannsen, A. (1965a) The

incidence of *Clostridium botulinum* type E in fish and bottom deposits in the North sea and off the coast of Scandinavia. *J. Appl. Bacteriol.* 28, 426-430.

Cardella, M.A., Duff, J.T., Wingfield, B.H. and Gottfried, C. (1960) Studies on immunity to toxins of *Clostridium botulinum.* VI Purification and detoxification of type D toxin and the immunological response to toxoid. *J. Bacteriol.* 79, 372-378.

Carroll, B.J., Garrett, E.S., Reese, G.B. and Ward, B.Q. (1966) Presence of *Clostridium botulinum* in the Gulf of Venenzuela and the Gulf of Darien. *Appl. Microbiol.* 14(5), 837-838.

Cartwright, T.E. and Lauffer, M.A. (1958). Temperature effects on botulinum A toxin. *Proc. Soc. Exptl. Biol. Med.* 98, 327-330.

CDC (Centres for Disease Control) (1979). *Botulism in the United States* (1899-1977). Handbook for Epidemiologists, Clinicians and Laboratory workers. U.S. Department of Health, Education and Welfare, CDC, Atlanta, GA.

CDC (Centre for Disease Control) (1987) International outbreak of type E botulism associated with ungutted, salted white fish. *Californian Morbidity Weekly Reports* 36, 812-813.

Chaibi, A., Ababouch, L.H., Belasri, K., Boucetta, S. and Busta, F.F. (1997). Inhibition of germination and vegetative growth of *Bacillus cereus* T, and *Clostridium botulinum* 62 A spores by essential oils. *Food Microbiol.* 14, 161-174.

Chapman, H.M. and Naylor, H.B. (1966) Isolation of *Clostridium botulinum* type E from Cayuga Lake fish. *Appl. Microbiol.* 14, 301-302.

Craig, J.M. and Pilcher, K.S. (1967) The natural distribution of *Clostridium botulinum* type E on the Pacific coast areas of the United States. In *"Botulism 1966"* (Eds. Ingram, M. and Roberts, T.A.) pp 56-61. London, Chapman and Hall.

Craig, J.M., Hayes, S. and Pilcher, K.S. (1968) Incidence of *Clostridium botulinum* type E in salmon and other marine fish in the Pacific North west. *Appl. Microbiol.* 16, 553-557.

Crandall, A.D. and Montville, T.J. (1993) Inhibition of *Clostridium botulinum* growth and toxigenesis in a model gravy system by coinoculation with bacteriocin producing lactic acid bacteria. *J. Food Protect.* 56, 485-488.

Crisley, F.D. (1960) Routine method for gold fish assay of toxin in crude culture centrifugates of *Clostridium botulinum* type A. *Appl. Microbiol.* 8, 282-285.

Davies, J.A. (1969) Isolation and identification of clostridia from North sea sediments. *J. Appl. Bacteriol.* 32, 164-169.

Dodds, K.L. (1989) Combined effect of water activity and pH on inhibition of toxin production by *Clostridium botulinum* in cooked vacuum packed potatoes. *Appl. Environ. Microbiol.* 55(3), 656-660.

Dodds, K.L. (1993 a) *Clostridium botulinum* in the environment. In *"Clostridium botulinum: Ecology and control in foods* (Eds. Hauschild, A.H.W. and Dodds, K.L.) pp 21-52. Inc, New York, Marcel Dekker.

Dodds, K.L. (1993 b) *Clostridium botulinum* in foods. In *"Clostridium botulinum: Ecology and control in foods.* (Eds. Hauschild, A.H.W. and Dodds, K.L.) pp 53-68. Inc, New York, Marcel Dekker.

Dodds, K.L., Brodsky, M.H. and Warburton, D.W. (1992) A retail survey of smoked ready to eat fish to determine their microbiological quality. *J. Food Protect.* 55(3), 208-210.

Dolman, C.E. (1957) Type E (Fish-borne) botulism: A review. *Jap. J. Med. Sci. Biol.* 10, 383-395.

Dolman, C.E. (1964) Botulism as a world health problem. In *"Botulism 1964"* (Eds. Lewis, K.H. and Cassel, K. Jr.) pp 5-32. Cincinnati, Ohio, U.S.Dept. of Health, Education and Welfare, Public Health Service.

Dolman, C.E. and Iida, H. (1963) Type E botulism: its epidemiology, prevention and specific treatment. *Can. J. Public Health* 54, 293-308.

Dolman, C.E., Darby, G.E. and Lane, R.F. (1961) *Clostridium botulinum* type F with recent observation on other types. *J. Infect. Dis.* 109, 107-128.

Dowell, V.R. Jr., Lombard, G.L., Thompson, F.S. and Armfield, A.Y. (1981) *"Media for isolation, characterisation and identification of obligately anaerobic bacteria".* U.S. Department of Health, Education, Welfare, Public Health Service, Centre for Diseases Control, Atlanta, Ga.

Eklund, M.W. (1982) Significance of *Clostridium botulinum* in fishery products preserved short of sterilization. *Food Technol.* 36(12), 107-112, 115.

Eklund, M.W. (1993) Control in fishery products. Chap. 7. In "*Clostridium botulinum*: *Ecology and Control in foods"* (Eds. Hauschild, A.H.W. and Dodds, K.L.) Inc, New York, Marcel Dekker.

Eklund, M.W. and Poysky, F. (1965) *Clostridium botulinum* type F from marine sediments. *Science*. 149, 306.

Eklund, M.W. and Poysky, F. (1967) Incidence of *Clostridium botulinum* type E from the Pacific coast of the United States. In *"Botulism 1966"* (Eds. Ingram, M. and Roberts, T.A.) p 49. London, Chapman and Hall.

Eklund, M.W. and Poysky, F.T. (1981) Relationship of bacteriophages to the toxigenicity of *Clostridium botulinum* and closely related organisms. In *Biomedical Aspects of botulism* (Eds. Lewis, G.E.Jr.) pp. 93-107. London, Academic Press.

Eklund, M.W., Petersen, M.E., Poysky, F.T., Peck, L.W. and Conrad, J.F. (1982) Botulism in juvenile cohosalmon (*Oncorhynchus kisutch*) in the United States. *Aquaculture* 27, 1-11.

Eklund, M.W., Poysky, F.T. and Wieler, D.I. (1967 b) Characteristics of *Clostridium botulinum* type F isolated from the Pacific coast of the United States. *Appl. Microbiol.* 15, 1316-1323.

Eklund, M.W., Poysky, F.T., Petersen, M.E., Peck, L.W. and Brunson, W.D. (1984) Type E Botulism in salmonids and conditions contributing to outbreaks. *Aquaculture*. 41, 293-309.

Eklund, M.W., Wieler, D.I. and Poysky, F.T. (1967 a) Outgrowth and toxin production of non-proteolytic type B *Clostridium botulinum* at 3.3 to 5.6°C. *J. Bacteriol.* 93, 1461-1462.

Emodi, A.S. and Lechowich, R.V. (1969 a) Low temperature growth of type E *Clostridium botulinum* spores. I. Effects of sodium chloride, sodium nitrite and pH. *J. Food Sci.* 34, 78-81.

Emodi, A.S. and Lechowich, R.V. (1969 b) Low temperature growth of type E

Clostridium botulinum spores. 2. Effects of solutes and incubation temperature. *J. Food. Sci.* 34, 82-87.

Eyles, M.J. and Warth, A.D. (1981) Assessment of the risk of botulism from vacuum-packaged raw fish: a review. *Food Technol. in Australia.* 33(11), 574-578.

Fantasia, L.D. and Duran, A.P. (1969) Incidence of *Clostridium botulinum* type E in commercially and laboratory dressed white fish chubs. *Food Technol. (Champaign).* 23, 793-794.

FAO (1996) List of animal species used in aquaculture. *FAO Fish. Cir. 914*, Rome, FAO.

FAO (1997) Aquaculture production statistics 1986-1995. *FAO Fish. Circ.* 815 Revision, 9, Rome, FAO.

Farber, J.M. (1991) Microbiological aspects of modified atmosphere packaging technology - A review. *J. Food Protect.* 54(1), 58-70.

Finne, G. (1982) Modified and Controlled atmosphere storage of muscle foods. *Food Technol.* 36, 128-133.

Fletcher, G.C., Murrell, W.G., Statham, J.A., Stewart, B.J. and Bremner, H.A. (1988) Packaging of Scallops with sorbate: An assessment of the hazard from *Clostridium botulinum J. Food Sci.* 53(2), 349-352, 358.

Fonseka, T.S.G. (1990) Microbial flora of pond cultured prawn *(Penaeus monodon). FAO Fish. Rep.* 401, 24-25.

Food and Drug Administration (1995) *Bacteriological Analytical Manual* 8th edn. Gaithersburg, MD, AOAC International.

Fryer, J.L. and Rohovec, J.S. (1993) Bacterial Diseases of fish. In *Pathobiology of Marine and estuarine organisms*. (Eds. Couch, J.A. and Fournie, J.W.) pp 53-83. Inc. London, CRC Press.

Garcia, G. and Genigeorgis, C. (1987) Quantitative evaluation of *Clostridium botulinum* non-proteolytic types B, E and F growth risk in fresh salmon tissue homogenates stored under modified atmospheres. *J. Food Protect.* 50(5), 390-397.

Garcia, G., Genigeorgis, C. and Lindroth, S. (1987) Risk of *Clostridium botulinum* non-proteolytic types B, E and F growth and toxin production in salmon fillets stored under modified atmospheres at low and abused temperatures. *J. Food Protect.* 50, 330-336.

Garren, D.M., Harrison, M.A. and Huang, Y.W. (1994) *Clostridium botulinum* type E outgrowth and toxin production in vacuum-skin packaged shrimp. *Food Microbiol.* 11(6), 467-472.

Gaze, J.E. (1992) The importance and control of *Clostridium botulinum* in processed foods. *British Food Journal.* 94(1), 8-15.

Genigeorgis, C. and Riemann, H. (1979) Food Processing and hygiene. In *Food-borne infections and intoxications* 2nd Edn. (Eds. Riemann, H. and Bryan, F.) pp. 613-713. New York, Academic Press.

Genigeorgis, C.A. (1985) Microbial and safety implications of the use of modified atmospheres to extend the storage of fresh meat and fish: A review. Int. *J. Food Microbiol.* 1, 237-251.

Georgala, D.L. and Hurst, A. (1963) The survival of food poisoning bacteria in frozen foods. *J. Appl. Bacteriol.* 26(3), 346-358.

George Joseph, K., Muraleedharan, V., Kalaimani, N. and Unnikrishnan Nair, T.S. (1986) Quality of cured fish from Tamilnadu coast. *Fish. Technol.* 23(1), 63-65.

George Joseph, K., Muraleedharan, V., Unnikrishnan, T.S. and Kalaimani, N. (1988) Quality of cured fish from the Maharashtra coast. *Fish Technol.* 25(2), p. 120-123.

Gibbs, P.A., Davies, A.R. and Fletcher, R.S. (1994) Incidence and growth of psychrotrophic *Clostridium botulinum* in foods. *Food Control* 5(1), 5-7.

Gibson, A.M., Bratchell, N. and Robert, T.A. (1987) The effect of sodium chloride and temperature on the rate and extent of growth of *Clostridium botulinum* type A in pasteurised pork slurry. *J. Appl. Bacteriol.* 62, 479-490.

Gibson, D.M. (1990) Pathogenic Bacteria in chilled foods. In *Processing and Quality of Foods.* Vol. 3. *Chilled Foods. The revolution in freshness* (eds. Zenthen, P., Cheftel, J.C., Eriksson, C., Gormley, T.R., Linko, P. and Paulus, K.) pp. 3.53-3.67. London, Elsevier Applied Science.

Goldblith, S.A. and Nickerson, J.T.R. (1965) The effect of gamma rays on haddock and clams inoculated with *Clostridium botulinum* type E. *U.S.Atomic Energy Commission Report No. 22*, Massachusetts Institute of technology. MIT 3325.

Goldmintz, D., Baninchak, J.A., Richards, G.P. and Graikoski, J.A. (1983) Bacteriological evaluation of steam pasteurized oysters, *Crassostrea virginica*. *Dev. Ind. Microbiol.* 24, 457-466.

Gopakumar, K. (1998). Risk associated with contaminated fish from aquaculture and control measures. Paper presented at the National Symposium on sutainable

aquaculture organised by Society of Ocean Scientists and Technologists and Department of Botany, University of Delhi on February 20-21, 1998, New Delhi.

Gopakumar, K. and Devadasan K. (1983) Fish curing industry in India. FAO Report *R.279 Supplement* p. 63-68.

Gopal, T.K.S., Nambiar, V.N., Bhattacharya, S.K., Jose Joseph and Prabhu, P.V. (1990) Modified atmosphere storage of freshwater fish fillets (*Catla catla*). In *Proceedings of the Symposium on chilling and freezing of new fish products.* pp 141-146. London, International Institute of Refrigeration.

Gopal, T.K.S., Nambiar, V.N., Lalitha, K.V., Jose Joseph and Prabhu, P.V. (1996) Modified atmosphere storage of fillets from sea bream *Argyrozona argyrozona.* In *Proc. Second Workshop Scient. Result: FORV Sagar Sampada.* pp 513-519. (Ed. Pillai, V.K., Abidi, S.A.H., Ravindranathan, V., Balachandran, K.K. and Agadi, V.I.) India, Dept. Ocean Development.

Graham, A.F. and Lund, B.M. (1986) The effect of citric acid on growth of proteolytic strains of *Clostridium botulinum J. Appl. Bacteriol.* 61, 39-49.

Graham, A.F. and Lund, B.M. (1987) The combined effect of sub-optimal temperature and sub-optimal pH on growth and toxin formation from spores of *Clostridium botulinum. J. Appl. Bacteriol.* 63, 387-393.

Graham, A.F. and Lund, B.M. (1993) The effect of temperature on the growth of non-proteolytic type B *Clostridium botulinum. Lett. Appl. Microbiol.* 16, 158-160.

Graham, A.F., Mason, D.R. and Peck, M.W. (1996) Inhibitory effect of combinations of heat treatment, pH and sodium chloride on growth from spores of non-proteolytic

C. botulinum at refrigeration temperature. *Appl. Environ. Microbiol.* 62(7), 2664-2668.

Grant, I.R., Ball, H.J. and Rowe, M.T. (1996) Thermal inactivation of several Mycobacterium spp. in milk by pasteurisation. *Lett. Appl. Microbiol.* 22(3), 253-256.

Haagsma, J. (1975) Sensitivity of eels (*Anguilla anguilla*) and carp (*Cyprinus carpio*) to type C and E botulinum toxin. *Zbl. Bakt. Hyg. Abt. Orig. A.* 230, 59-66.

Halbinger, R.E. (1973) *Clostridium botulinum* in fishery products. In *Microbial Safety of fishery products.* (Eds. Chichester, C.O. and Graham, H.D.) pp 191-202. London, Academic Press.

Haq, I. and Sakaguchi, G. (1980) Prevalence of *Clostridium botulinum* in fishes from markets in Osaka. *Japan J. Med. Sci. Biol.* 33, 1-6.

Haq, I. and Suhadi, F. (1981) Incidence of *Clostridium botulinum* in coastal and Inland areas of West Java. *Japan J. Med. Sci. Biol.* 34, 231-235.

Harrigan, W.F. and McCance, M.E. (1976) *Laboratory methods in Food and Dairy Microbiology* pp.452. London: Academic Press, Inc.

Hatheway, C.L. (1993) *Clostridium botulinum* and other Clostridia that produce botulinum neurotoxin. In *Clostridium botulinum: Ecology and control in foods* (Eds. Hauschild, A.H.W. and Dodds, K.L.) pp. 3-20, New York. Marcel Dekker.

Hauschild, A.H.W. (1989) *Clostridium botulinum.* In *Food-borne bacterial pathogens* (Ed. Doyle, M.P.) pp. 111-189, New York, Marcel Dekker.

Hauschild, A.H.W. (1993) Epidemiology of human food borne botulism. In *Clostridium botulinum*: *Ecology and control in foods*. (Eds. Hauschild, A.H.W. and Dodds, K.L.) pp 69-104. New York, Marcel Dekker.

Hauschild, A.H.W., Hilsheimer, R., Jarvis, G. and Raymond, D.P. (1982) Contribution of nitrite to the control of *Clostridium botulinum* in liver sausage. *J. Food Protect.* 45, 500-506.

Hayashi, R., Tokuchi, M., Teramoto, K., Fujita, N., Okuno, Y., Rahim, A., Sujudi, R. and Hotta, S. (1981) Distribution of *Clostridium botulinum* in Indonesian soil. In *I.C.M.R. Annals* (Eds. Hotta, S.) Vol. I pp 187-193. Kobe, Int. Cent. For Med. Res., Kobe Univ. Sch. Med.

Hayashi, T., Kamiya, S., Yoshimura, K. and Nakamura, S. (1990) *Clostridium botulinum* in the soil of Paraguay. *Japan J. Med. Sci. Biol.* 43(1), 19-21.

Hayes, S., Craig, J.M. and Pilcher, K.S. (1970) The detection of *Clostridium botulinum* type E in smoked fish products in the Pacific North west. *Can. J. Microbiol.* 16, 207-209.

Hiroki, H. (1970) Sensitivity and immune response of freshwater fishes to *Clostridium botulinum* type E toxin. In *Toxic Microorganisms* (Ed. Herzberg, M.) pp 380-383. Washington, D.C, U.S. Dept. of the Interior.

Hobbs, G. (1976) *Clostridium botulinum* and its importance in fishery products. In *Advances in Food Research* Vol.22. (Ed. Chichester, C.O.) pp 135-185. London, Academic Press.

Hobbs, G. (1981) The Ecology and Taxonomy of Psychrotrophic strains of *Clostridium botulinum*. In *Psychrotrophic microorganisms in spoilage and pathogenicity.*

(Eds. Roberts, T.A., Hobbs, G., Christian, J.H.B. and Skovgaard, N.) pp 449-462. London, Academic Press.

Hobbs, G., Can, D.C., Wilson, B.B. and Shewan, J.M. (1965) The incidence of organisms of the genus *Clostridium* in vacuum packed fish in the United Kingdom. *J. Appl. Bacteriol.* 28, 265.

Horsley, R.W. (1973) The bacterial flora of the Atlantic salmon (Salmo salar, L) in relation to its environment. *J. Appl. Bacteriol.* 50, 359-377.

Hotchkiss, J.H. (1988) Experimental approaches to determining the safety of food packaged in modified atmospheres. *Food Technol.* 42, 55-64.

Houghtby, G.A. and Kaysner, C.A. (1969) Incidence of *Clostridium botulinum* type E in Alaskan salmon. *Appl. Microbiol.* 18, 950-951.

Hubalek, Z. and Halouzka, J. (1988) Thermal sensitivity of *Clostridium botulinum* type C toxin. *Epidemiol. Infect.* 101(2), 321-325.

Huhtanen, C.N., Naghski, J., Custer, C.S. and Russell, R.W. (1976) Growth and toxin production by *Clostridium botulinum* in moldy tomato juice. *Appl. Environ. Microbiol.* 32, 711-715.

Huss, H.H. (1980) Distribution of *Clostridium botulinum*. *Appl. Environ. Microbiol.* 39(4), 764-769.

Huss, H.H. (1981 a) The Ecology of *Clostridium botulinum* type E. In *Psychrotrophic Microorganisms in spoilage and pathogenicity.* (Eds. Roberts, T.A., Hobbs, G., Christian, J.H.B. and Skovgaard, N.) pp 463-473. London, Academic Press.

Huss, H.H. (1981 b) *Clostridium botulinum type E and botulism.* 58 pp. Lyngby, Denmark, Technical Univ. of Denmark.

Huss, H.H. (1994). Assurance of seafood quality. *FAO Fisheries Technical Paper* 334, FAO, Rome.

Huss, H.H. and Eskildsen, U. (1974). Botulism in farmed trout caused by *Clostridium botulinum type E. Vet. Med.* 26, 733-738.

Huss, H.H. and Pedersen, A. (1979) *Clostridium botulinum* in fish. *Nord. Vet. Med.* 31 (5), 214-221.

Huss, H.H. and Petersen, E.R. (1980) The stability of *Clostridium botulinum* type E toxin in salty and/or acid environment. *J. Food Technol.* 15(6), 619-627.

Huss, H.H., Pedersen, A. and Cann, D.C. (1974 a). The incidence of *Clostridium botulinum* in Danish trout farms.I. Distribution in fish and their environment. *J. Food Technol.* 9, 445-450.

Huss, H.H., Pedersen, A. and Cann, D.C. (1974 b). The incidence of *Clostridium botulinum* in Danish trout farms. II. Measures to reduce contamination of the fish. *J. Food Technol*, 9, 451-458.

Huss, H.H., Schaeffer, I., Pedersen, A. and Jepsen, A. (1979 b) Toxin production by *Clostridium botulinum* type E in smoked fish in relation to the measured oxidation reduction potential (Eh), packaging method and the associated microflora. In *Advances in Fish Science and Technology.* pp 477-479. Farnham, Surrey, England, Fishing News (Books) Ltd.

Huss, H.H., Schäeffer, I., Pedersen, A. and Jepsen, A. 1979a. Toxin production by *Clostridium botulinum* type E in fresh herring in relation to the measured oxidation reduction potential (Eh). *Nord. Vet. Med.* 31, 81-86.

ICMSF. (1980) *Microbial Ecology of foods* Vol. I. *Factors affecting life and death of Microorganisms*. International Commission on Microbiological specification of foods, Inc. London, Academic Press.

Ikawa, J.Y. and Genigeorgis, C. 1987. Probability of growth and toxin production by non-proteolytic *Clostridium botulinum* in rock fish fillets stored under modified atmospheres. *Int. J. Food Microbiol.* 4, 167-181.

Inglis, V., Roberts, R.J. and Bromage, N.R. (1993) *Bacterial Diseases of fish*. New York: Halsted Press.

Insalata, N.F., Fredericks, G.J., Berman, J.H. and Borker, E. (1967) *Clostridium botulinum* type E in frozen vacuum-packed fish. *Food Technol.* 21, 296-298.

IS 2882 (1964) *Specification for dried white baits* (*Anchoviella* sp.) Indian Standards Institution, New Delhi.

IS 5198. (1969) *Specification for dry salted seer fish*. Indian Standards Institution, New Delhi.

IS. 2883 (1976) *Specification for dried white baits*. Indian Standards Institution, New Delhi.

IS. 594 (1962) *Specification for common salt for fish curing (revised)*. Indian Standards Institution, New Delhi.

Ito, K.A. and Chen, J.K. (1978) Effect of pH on growth of *Clostridium botulinum* in foods. *Food Technol (Champaign)*. 32(6), 71-72, 76.

Jarvis, B. and Patel, M. (1980) The occurrence and control of *Clostridium botulinum* in foods. In *Food and Health Science and Technology*. (Eds. Birch, G.G. and Parker, K.J.) pp 101-114. England, Applied Science Publishers Ltd.

Jensen, M.J., Genigeorgis, C. and Lindroth, S. 1987. Probability of growth of *Clostridium botulinum* as affected by strain, cell and serological type, inoculum size and temperature and time of incubation in a model broth system. *J. Food Safety*. 8, 109-126.

Johannsen, A. (1965 a) *Clostridium botulinum* type E in foods and the environment generally. *J. Appl. Bacteriol.* 28, 90-94.

Johannsen, A. (1965 b) *Clostridium botulinum* type E. I. Smoked fish products. *Sartrycksv.* Vet. 5, 1.

Johnston, R.W., Feldman, J. and Sullivan, R. (1963) Botulism from canned tuna fish. *Public Health Reports*. 78, 561-564.

Kalaimani, N., Gopakumar, K. and Unnikrishnan Nair, T.S. (1988) Quality characteristics of cured fish of Commerce. *Fish Technol.* 25(1), 54-57.

Kamiya, S., Yoshimura, K., Nakamura, S. and Ezaki, T. (1990) *Clostridium botulinum* in the soil of Kenya. *Ann. Trop. Med. Parasitol.* 84(2), 201-203.

Kanzawa, K., Ono, T., Karashimada, T. and Iida, H. (1970) Distribution of *Clostridium botulinum* type E in Hokkaido, Japan. In *Toxic Microorganisms*. (Ed. Hertzberg, M.) pp 309-316. Washington, D.C, U.S. Dept. Interior.

Lalitha, K.V. and Iyer, K.M. (1986) Incidence of *Clostridium perfringens* in fishes. *Fish. Technol.* 23, 152-157.

Lalitha, K.V. and Iyer, K.M. (1990) Isolation of *Clostridium botulinum* from tropical fish. *Letters in Appl. Bacteriol.* 11, 179-181.

Lalitha, K.V. and Surendran, P.K. (1992) Prevalence of *Clostridium botulinum* in fresh retail fish. In *Proceedings of the fourth Kerala Science Congress* (Ed.

Ramachandran Nair, C.G.) p 117-118. Thiruvananthapuram, State Committee on Science, Technology and Environment, Government of Kerala, S.B. Press.

Lalitha, K.V. and Surendran, P.K. (1993) Isolation of toxigenic strains of *Clostridium botulinum* type D from aquatic environment. In *Nutrients and bioactive substances in aquatic organisms.* (Eds. Devadasan, K., Mukundan, M.K., Antony, P.D., Viswanathan Nair, P.G., Perigreen P.A. and Jose Joseph). Pp 144-149. Cochin, India, Society of Fisheries Technologists (India).

Lalitha, K.V., Unnithan, G.R. and Iyer, K.M. 1990. Distribution of *Clostridium perfringens* in fishes and prawns. *Fish. Technol.* 27, 64-68.

Lerke, P. (1973) Evaluation of potential risk of botulism from seafood cocktails. *Appl. Microbiol.* 25(5), 807-810.

Lerke, P. and Farber, L. (1971) Heat pasteurisation of crab and shrimp from the Pacific coast of the United States: Public Health aspects. *J. Food Sci.* 36, 277-279.

Lewis, G.E. Jr. 1981. *Biomedical Aspects of Botulism.* Academic Press, Inc. London.

Licciardello, J.J. (1983). Botulism and heat-processed seafoods. *Marine Fish. rev.* 45(2), 1-7.

Licciardello, J.J., Nickerson, J.T.R., Ribich, C.R. and Goldblith, S.A. (1967 b) Thermal inactivation of type E botulinum toxin. *Appl. Microbiol.* 15, 249-256

Licciardello, J.J., Ribich, C.A., Nickerson, J.T.R. and Goldblith, S.A. (1967a) Kinectics of the thermal inactivation of type E *Clostridium botulinum* toxin. *Appl. Microbiol.* 15, 344-349.

Lilly, T, Jr. And Kautter, D.A. (1990) Outgrowth of naturally occurring *Clostridium botulinum* in vacuum packaged fresh fish. *J. Assoc. off. Anal. Chem.* 73, 211-212.

Lindroth, S.E. and Genigeorgis, C.A. (1986) Probability of growth and toxin production by non-proteolytic *Clostridium botulinum* in rock fish stored under modified atmospheres. *Int. J. Food Microbiol.* 3, 167-187.

Liston, J. (1990) Microbial hazards of sea food consumption. *Food Technol.* 44, 56-62.

Llobrera, A.T. 1983. Bacteriological safety assessment of *Clostridium botulinum* in fresh fish and shellfish packaged in modified atmosphere containing CO_2. Ph.D. thesis, Texas A and M University, College Station Tx.

Llobrerra, T.A., Bulalacao, L.M. and Tan, A. (1990) Effect of farming phase and in plant processing on the microbiological quality of prawns (*Penaeus monodon*). *FAO Fish. Rep.* 401: FAO, Rome, pp 1-5.

Losikoff, M.E. (1978) Establishment of a heat inactivation curve for *Clostridium botulinum* 62A toxin in beef broth. *Appl. Environ. Microbiol.* 36, 386-388.

Lund, B.M. (1986) Anaerobes in relation to foods of plant origin. In *Anaerobic bacteria in habitats other than man*" (Eds. Barnes, E.M. and Mead, G.C.) pp 351-372. Oxford, London, Blackwell Scientific Publications.

Lund, B.M. (1993) Quantification of factors affecting the probability of development of pathogenic bacteria, in particular *Clostridium botulinum* in foods. In Application of Predictive Microbiology and computer modelling techniques to the Food industry. *J. Industrial Microbiol.* 12, 144-155.

Lund, B.M. and Peck, M.W. 1994. Heat resistance and recovery of spores of non-proteolytic *Clostridium botulinum* in relation to refrigerated, processed foods with an extended shelf-life. *J. Appl. Bacteriol. Symposium supplement* 76, 115S-128S.

Lund, B.M. and Wyatt, G.M. (1984) The effect of redox potential, and its interaction with sodium chloride concentration on the probability of growth of *Clostridium botulinum* type E from spore inocula. *Food Microbiol.* 1, 49-65.

Lund, B.M., Graham, A.F. and Franklin, J.G. (1987) The effect of acid pH on the probability of growth of proteolytic strains of *Clostridium botulinum*. *Int. J. Food Microbiol.* 4, 215-226.

Lund, B.M., Graham, A.F., George, S.M. and Brown, D. (1990) The combined effect of incubation temperature, pH and sorbic acid on the probability of growth of non-proteolytic type B *C. botulinum*. *J. Appl. Bacteriol.* 69, 481-492.

Lund, B.M., Wyatt, G.M. and Graham, A.F. (1985) The combined effect of low temperature and low pH on survival and growth and toxin formation from spores of *Clostridium botulinum*. *Food Microbiol.* 2, 135-145.

Lynt, R.K., Kautter, D.A. and Solomon, H.M. (1982) Differences and similarities among proteolytic and non-proteolytic strains of *Clostridium botulinum* types A, B, E and F: A review. *J. Food Protect.* 45(5), 466-474.

Mac Faddin, J.F. 1980. *Biochemical tests for identification of Medical bacteria*. Second edition. Williams and Wilkins, Baltimore, U.S.A.

Marshall, B.J., Ohye, D.F. and Christian, J.H.B. (1971) Tolerance of bacteria to high concentrations of sodium chloride and glycerol in the growth medium. *Appl. Microbiol.* 21, 363-364.

Matches, J.R. and Liston, J. 1974. Mesophilic Clostridia in Puget Sound. *Can. J. Microbiol.* 20(1), 1-7.

McClure, P.J., Cole, M.B. and Smelt, J. P.P.M. (1994) Effects of Water activity and pH on growth of *Clostridium botulinum. J. Appl. Bacteriol. Symposium Supplement* 76, 105 S-114 S.

Montville, T.J. (1982) Metabiotic effects of *Bacillus licheniformis* on *Clostridium botulinum*: implications for home-canned tomatoes. *Appl. Environ. Microbiol.* 44, 334-338.

Mortojudo, J.W., Siagian, E.G., Suhadi, F., Ward, B.Q. and Ward, W.M.S. (1973) The presence of *Clostridium botulinum* in Indonesian Waters. *J. Appl. Bacteriol.* 36, 437-440.

Nayyarahamed, Karunasagar, I. and Karunasagar, I. (1994) Microbiology of cultured shrimps in India. *FAO Fish. Rep. 514 supplement*. 13-22.

Nedoluha, P.C. and Westhoff, D. (1993) Microbiological flora of aquacultured hybrid Striped Bass. *J. Food Protect.* 56(12), 1054-1060.

Nickerson, J.T.R., Goldblith, S.A., di Gioia, G. and Bishop, V.V. (1967) The presence of *Cl. botulinum* type E in fish and mud taken from the Gulf of Maine. In *"Botulism 1966"* (Eds. Ingram, M and Roberts, T.A.) p 25-33. London, Chapman and Hall Ltd.

Nielsen, S.F. and Pedersen, H.O.(1967) Studies on the occurrence and germination of *Cl. botulinum* in smoked salmon. In *"Botulism 1966"* (Eds. Ingram, M. and Roberts, T.A.) p 66. London, Chapman and Hall Ltd.

Notermans, S., Gallhoff, G., Zwietering, M.H. and Mead G.C. (1995) The HACCP concept: specification of criteria using quantitative risk assessment. *Food Microbiol.* 12, 81-90.

Notermans, S., Zwietering, M.H. and Mead, G.C. (1994) The HACCP concept: identification of potentially hazardous microorganisms. *Food Microbiol.* 11, 203-214.

Odlaug, T.E. and Pflug, I.J. (1978) *Clostridium botulinum* and Acid foods. *J. Food Protect.* 41(7), 566-573.

Odlaug, T.E. and Pflug, I.J. (1979) *Clostridium botulinum* growth and toxin production in tomato juice containing *Aspergillus gracilis*. *Appl. Environ. Microbiol.* 37(3), 496-501.

Ogbondeminu, F.S., Omorinkoba, W.S., Madu, C.T. and Ibikunle, G.P.(1991) Bacterial microflora associated with fish production in outdoor concrete ponds and their feed. *J. Aqua. Trop.* 6, 55-62.

Oguma, K., Yamaguchi, T., Sudou, K., Yokosawa, N. and Fujikawa, Y. (1986) Biochemical classification of *Clostridium botulinum* type C and D strains and their non-toxigenic derivatives. *Appl. Environ. Microbiol.* 51(2), 256-260.

Oguma, K., Yokota, K., Hayashi, S., Takeshi, K., Kumagai, M., Itoh, N., Tachi, N. and Chiba, S. (1990) "Infant botulism due to *Clostridium botulinum* type C toxin" *Lancet.* 336, 1449-1450.

Ohye, D.F. and Christian, J.H.B. (1967) Combined effects of temperature, pH and water activity on growth and toxin production by *Clostridium botulinum* type A, B and E. In *"Botulism 66"* (Eds. Ingram, M. and Roberts, T.A.) pp 217-223. London, Chapman and Hall Ltd.

Ohye, D.F. and Scott, W.J. (1953) The temperature relations of *Clostridium botulinum* types A and B. *Australian J. Biol. Sci.* 6, 178-189.

Ohye, D.F. and Scott, W.J. (1957) Studies on the physiology of *Clostridium botulinum* type E. *Aust. J. Biol. Sci.* 10, 85-94.

Okereke, A. and Montville, T.J. (1991) Bacteriocin-mediated inhibition of *Clostridium botulinum* spores by lactic acid bacteria at refrigeration and abuse temperatures. *Appl. Environ. Microbiol.* 57(12), 3423-3428.

Ortiz, N.E. and Smith, G.R. (1994) Landfill sites, botulism and gulls. *Epidemiol. Infect.* 112(2), 385-391.

Otofuji, T. Tokiwa, H. and Takahashi, K. (1987) A food poisoning incident caused by *Clostridium botulinum* toxin A in Japan. *Epidemiol. Infect.* 99, 167-172.

Owens, J.D. and Mendoza, L.S. 1985. Enzymically hydrolysed and bacteriologically fermented fishery products. *J. Food Technol.* 20, 273-293.

Pace, P.J., Krumbiegel, E.R., Angelloti, R. and Wisniewiski, H.T. (1967) Demonstration and isolation of *Clostridium botulinum* types from white fish chubs collected at fish smoking plants in the Milwankee area. *Appl. Microbiol.* 15, 877-884.

Palumbo, M.S., Beers, S.M., Bhaduri, S. and Palumbo, S.A. 1995. Thermal resistance of *Salmonella* spp. And *Listeria monocytogenes* in liquid egg yolk and egg yolk products. *J. Food Protect.* 58(9), 960-966.

Pasricha, C.L. and Panja, G. (1940) *Clostridium botulinum* in samples of Calcutta soil. *Indian J. Med. Res.* 28, 49-54.

Patel, H.R., Patel, P.C. and York, G.K. (1978) Growth and toxigenesis of *Clostridium botulinum* type E on marine molluscs at low temperatures. *J. Food Sci. and Technol.* 15(6), 231-235.

Peck, M.W., Lund, B.M., Fairbairn, D.A., Kaspersson, A.S. and Undeland, P.C. (1995) Effect of heat treatment on survival of, and growth from, spores of non-proteolytic *Clostridium botulinum* at refrigeration temperatures. *Appl. Environ. Microbiol.* 61(5), 1780-1785.

Penny, N., Bell, R.G. and Cummings, T.L. (1994) Extension of the chilled storage life of smoked blue cod (*Parapercis colias*) by carbon dioxide packaging. *Int. J. Food Science and Technology.* 29, 167-178.

Pillay, T.V.R. (1992) *Aquaculture and the environment.* Osney Mead, England: Fishing News books.

Post, L.S., Lee, D.A., Solberg, M.M., Furgang, D., Specchio, J. and Graham, C. (1985). Development of botulinal toxin and sensory deterioration during storage of vacuum and modified atmosphere packaged fish fillets. *J. Food. Sci.* 50, 990-996.

Prasad, M.M., Panduranga Rao, C.C. and Gupta, S.S. (1994) Chemical and Microbiological Quality of Dry fish from Kakinada. *Fish. Technol.* 31(1), 75-78.

Presnell, M.W., Miescier, I.J. and Hill, W.F.Jr. (1967) *Clostridium botulinum* in marine sediments and in the Oyster *(Crassostrea virginica)* from Mobile bay. *Appl. Microbiol.* 15, 668-669.

Prévot, A.R. and Brygoo, E.R. (1953) Nouvelles recherces sur le botulisme et ses cinq types toxiniques. *Ann. Inst. Past.* 85, 544-575.

Raatjes, G.J.M. and Smelt, J.P.P.M. (1979) *Clostridium botulinum* can grow and form toxin at pH lower than 4.6. *Nature* 281, 398-399.

Reddy, N.R., Armstrong, D.J., Rhodehamel, E.J. and Kautter, D.A. (1992) Shelf-life extension and safety concerns about fresh fishery products packaged under

modified atmospheres: A. review. *J. Food Safety* 12, 87-118.

Reddy, N.R., Paradis, A., Roman, M.G., Solomon, H.M. and Rhodehamel, E.J. (1996) Toxin development by *Clostridium botulinum* in Modified Atmosphere Packaged fresh Tilapia fillets during storage. *J. Food Sci.* 61(3), 632-635.

Reilly, A. and Käferstein, F. (1997) Food Safety hazards and the application of the principles of the hazard analysis and critical control point (HACCP) system for their control in aquaculture production. *Aquaculture Research* 28, 735-752.

Richard Molard, D. and Lesage, L. (1986) Food Microbiology and Water activity. In *Food Packaging and Preservation. Theory and Practice* (Ed. Mathlouthi, M.) pp. 165-180. London, Elsevier Applied Science Publishers Ltd.

Roberts, R.J. (1989) The Bacteriology of toleosts Chapter 8. In *Fish Pathology*. Second Edition. pp 289-319. London, Bailliere Tindall.

Roberts, T.A. and Gibson, A.M. (1979) The relevance of *Clostridium botulinum* type C in public health and food poisoning. *J. Food Technol.* 14(3), 211-226.

Roberts, T.A. and Hobbs, G. (1968) Low temperature growth characteristics of Clostridia. *J. Appl. Bacteriol.* 31, 75-88.

Roberts, T.A. and Ingram, M. (1973) Inhibition of growth of *Clostridium botulinum* at different pH values by sodium chloride and sodium nitrate. *J. Food Technol.* 8, 467-475.

Roberts, T.A. and Lund, B.M. (1991) Growth of *Clostridium botulinum* in foods. Paper presented at symposium held at 60th annual meeting of Society for Applied Bacteriology at Bristol, 15-19 July 1991.

Roberts, T.A. and Smart, J.L. (1976) The occurrence and growth of *Clostridium* spp. in vacuum-packed bacon with particular reference to *Clostridium perfringens* (*Welchii*) and *Clostridium botulinum*, *J. Food Technol.* 11, 229-244.

Roberts, T.A. and Thomas, J.A. (1982) Germination and outgrowth of single spores of *Clostridium botulinum* and putrefactive anaerobes. *J. Appl. Bacteriol.* 33, 313-321.

Rouhbakhsh-Khaleghdoust, A. (1975) The incidence of *Clostridium botulinum* type E in fish and bottom deposits in caspian sea coastal waters. *Pahlavi Medical Journal* 6, 550-556.

Sakaguchi, G. (1979) Botulism Chapter VIII. In *Food-borne Infections and Intoxications.* Second edition (Eds. Riemann, H. and Bryan, F.L.) pp 389-442. London, Academic Press.

Sakata, T., Sugita, H., Mitsuoka, T., Kakimoto, D. and Kadota, H. (1980) Isolation and distribution of Obligate anaerobic bacteria from the intestines of freshwater fish. *Nippon Suisan Gakkaishi.* 46(10), 1249-1255.

Schmidt, C.F., Lechowich, R.V. and Folinazzo, J.F. (1961) Growth and toxin production by type E *Clostridium botulinum* below 40°F. *J. Food Sci.* 26, 626-630,

Schofield, G.M. (1992) A Review: Emerging food-borne pathogens and their significance in chilled foods. *J. Appl. Bacteriol.* 72(4), 267-273.

Scott, W.J. (1950) Thermal destruction of type A *Clostridium botulinum* toxin. The nature of the protective substances in canned vegetables. *Aust. J. Appl. Sci.* 1, 200.

Scott, W.J. and Stewart, D.F. (1953) The thermal destruction of *Clostridium botulinum* toxin in canned vegetables. *Aust. J. Appl. Sci.* 1, 188-199.

Segner, W.P., Schmidt, C.F. and Boltz, J.K. (1966) Effect of sodium chloride and pH on the outgrowth of spores of type E *Clostridium botulinum* at optimal and suboptimal temperatures. *Appl. Microbiol.* 14, 49-54.

Segner, W.P., Schmidt, C.F. and Boltz, J.K. (1971 a) Enrichment, isolation and cultural characteristics of marine strains of *Clostridium botulinum* type C. *Appl. Microbiol.* 22(6), 1017-1024.

Segner, W.P., Schmidt, C.F. and Boltz, J.K. (1971 b) Minimal growth temperature, sodium chloride tolerance, pH sensitivity and toxin production of marine and terrestrial strains of *Clostridium botulinum* type C. *Appl. Microbiol.* 22(6), 1025-1029.

Sharmila, R., Abraham, T.J. and Sundararaj, V. (1996) Bacterial flora of semi intensive pond reared *Penaeus indicus* (H. Milne Edwards) and the environment. *J. Aqua. Trop.* 11, 193-203.

Shewan, J.M. (1967) The microbiology of fish spoilage and preservation. *Progress in Industrial Microbiology.* 6, 169-208.

Shewan, J.M. (1977) The bacteriology of fresh and spoiling fish and the biochemical changes induced by bacterial action. In *"Proc. Conf. Handl. Proc. Mark. Trop. Fish.* pp 5-9. London, Tropical Products Institute.

Siegel, L.S. (1981) Fermentation kinetics of botulinum toxin production (Types A, B and E). In *Biomedical aspects of botulism* (Ed. Lewis, G.E. Jr.) pp 121-128. New York, Academic Press.

Siegel, L.S. and Metzger, J.F. (1979) Toxin production by *Clostridium botulinum* type A under various fermentation conditions. *Appl. Environ. Microbiol.* 38(4), 606-611.

Siegel, L.S. and Metzger, J.F. (1980) Effect of fermentation conditions on toxin production by *Clostridium botulinum* type B. *Appl. Environ. Microbiol.* 40(6), 1023-1026.

Sindermann, C.J. (1990 a) *Principal Diseases of Marine fish and shellfish*: II edition. Vol. I. *Diseases of marine fish.* San Diego California, Academic Press.

Sindermann, C.J. (1990 b) *Principal Diseases of Marine fish and shellfish.* II edition. Vol. II. *Diseases of marine shellfish.* San Diego, California, Academic Press.

Sivakami, R., Premkishore, G. and Chandran, M.R. (1996) Occurrence and distribution of potentially pathogenic Enterobacteriaceae in carps and pond water in Tamil Nadu, India. *Aquaculture Research.* 27(5), 375-378.

Skulberg, A. and Grande, M. (1967) Susceptibility of rainbow trout *Salmo gairdneri* Richardson to *Clostridium botulinum* toxins. *Transact. Amer. Fish. Soc.*96, 67-70.

Slater, P.F., Addiss, D.G., Cohen, A., Levanthal, A., Chassis, G., Zehavi, H., Bashari, A. and Costin, C. (1989) Food borne botulism: an international outbreak. *International J. Epidemiol.* 18, 693-696.

Smelt, J.P.P.M. and Has, H. (1978) Behaviour of proteolytic *Clostridium botulinum* types A and B near the lower temperature limits of growth. *Eur. J. Appl. Microbiol. Biotechnol.* 5(2), 143-154.

Smelt, J.P.P.M., Raatjes, G.J.M., Crowther, J.S. and Verrips, C.T. (1982) Growth and toxin formation by *Clostridium botulinum* at low pH values. *J. Appl. Bacteriol.* 52, 75-82.

Smith, G.R. (1987) Botulism in water birds and its relation to comparative medicine. In *Avian Botulism - An international Perspective* (Eds. Eklund, M.W. and Dowell,

V.R.Jr.) pp 73-86. Springfield, Illinois, U.S.A., Charles C. Thomas.

Smith, G.R. (1990) Botulism. In *Topley and Wilison's Principles of Bacteriology, Virology and Immunity.* Vol. 3, *Bacterial Diseases* (Eds. Smith G.R. and Easman, C.S.F.) pp.514-529. London Edward Arnold.

Smith, G.R. and Young, A.M. (1980) *Clostridium botulinum* in British soil. *J. Hygiene.* 85, 271-274.

Smith, L.D.S. (1975) Common mesophilic anaerobes, including *Clostridium botulinum* and *Clostridium tetani* in 21 soil specimens. *Appl. Microbiol.* 29(5), 590-594.

Sneath, P.H.A. (1986) Genus *Clostridium.* In *Bergey's Manual of Systematic Bacteriology.* Vol. 2. (Ed. Sneath, P.H.A., Mair, N.S., Sharpe, M.E. and Holt, J.G.) pp 1141-1200. Baltimore, The Williams and Wilkins Co.

Solomon, H.M., Kautter, D.A. and Lynt, R.K. (1982) Effect of low temperatures on growth of non-proteolytic *Clostridium botulinum* types B and F and proteolytic types G in crab meat and broth. *J. Food Protect.* 45, 516-518.

Solomon, H.M., Lynt, R.K., Lilly, T. Jr. and Kautter, D.A. (1977) Effect of low temperature on growth of *Clostridium botulinum* spores in meat of the blue crab. *J. Food Protect.* 40, 5-7.

Solomon, H.M., Rhodehamel, E.J. and Kautter, D.A. (1995) *Clostridium botulinum* Chap. 17. In. *FDA Bacteriological Analytical Manual.* 8th edition. p. 17.01-17.10. AOAC international Gaithersburg MD 20877, USA.

Sonnabend, O. and Sonnabend, W. (1981) Different types of *Clostridium botulinum* (A, D and G) found at autopsy in humans: II. Pathological and epidemiological findings in twelve sudden and unexpected deaths, In *Biomedical aspects of*

botulism (Ed. Lewis, G.E. Jr.) pp 303-316. New York, Academic Press.

Sperber, W.H. (1982) Requirements of *Clostridium botulinum* for growth and toxin production. *Food Technol.* 36(12), 89-94.

Stier, R.F., Bell, L., Ito, K.A., Shafer, B.D., Brown, L.A., Seeger, M.L., Allen, B.H., Porcuna, M.N. and Lerke, P.A. (1981) Effects of modified atmosphere storage on *Clostridium botulinum* toxigenesis and the spoilage microflora of salmon fillets. *J. Food Sci.* 46, 1639-1642.

Strasdine, G.A. and Kelly, J.M. (1967) Germination of spores of *Clostridium botulinum* type E in fish and shellfish extracts. *J. Fish. Res. Bd. Canada.* 24(8), 1833-1835.

Strickland, J.D.H. and Parsons T.R. (1968). *A practical handbook of seawater analysis.* Ottawa, Fisheries Research Board of Canada.

Strom, M.S., Eklund, M.W. and Poysky, F.T. (1984) Plasmids in *Clostridium botulinum* and related clostridium species. *Appl. Environ. Microbiol.* 48(5), 956-963.

Sugita, H., Arai, H., Okada, S., Nagaya, M. and Deguchi, Y. (1989) Changes of the bacterial composition of gold fish culture water during the decomposition of food pellets. *Nippon Suisan Gakkaishi.* 55(4), 661-668.

Sugita, H., Miyajima, C., Iwata, J., Arai, S., Kubo, T., Igarashi, S. and Deguchi, Y. (1988) Intestinal microflora of Japanese coastal fishes. *Nippon Suisan Gakkaishi.* 54(5), 875-882.

Sugita, H., Tokuyama, K. and Deguchi, Y. (1985) The intestinal microflora of Carp *Cyprinus carpio,* Grass carp *Ctenopharyngodon idella,* and Tilapia *Sarotherodon niloticus. Bull. Jap. Soc. Sci. Fish.* 51(8), 1325-1329.

Sugiyama, H. (1980) *Clostridium botulinum* neurotoxin. *Microbiol. Rev.* 44, 419-448.

Sugiyama, H., Bott, T.L. and Foster, E.M. (1970) *Clostridium botulinum* type E in an inland bay (Green Bay of Lake michigan) In *Toxic Micro-organisms* (Ed. Herzberg, M.) pp 287-291. Washington, D.C., U.S. Dept. of the Interior.

Suhadi, F., Thayib, S.S. and Sumarsono, N. (1981) Distribution of *Clostridium botulinum* around fishing areas of the western part of Indonesian waters. *Appl. Environ. Microbiol.* 41(6), 1468-1471.

Surendran, P.K., Thampuran, N. and Gopakumar, K. (1994) Microbial profile of cultured fishes and prawns viz. A viz. Their spoilage and contamination. *FAO Fish. Rep. 514. Supplement*, FAO, Rome, pp 1-12.

Takano, M., Sakata, T. and Kakimoto, D. (1984) Microflora of bottom muds in the Kotsuki river and Kinko bay. *Mini review data file fisheries Research*, Kagoshima University, 3, 63-66.

Tanaka, N. (1982). Toxin production by *Clostridium botulinum* in media at pH lower than 4.6. *J. Food Protect.* 45, 234-237.

Tanasugarn, L. (1979) *Clostridium botulinum* in the Gulf of Thailand. *Appl. Environ. Microbiol.* 37(2), 194-197.

Telzak, E.E., Bell, E.P. and Shultz, S. (1990) An International outbreak of type E botulism due to uneviscerated fish. *J. Infect. Dis.* 161, 340-342.

Thatcher, F.S., Erdman, I.E. and Pontefract, R.D. (1967) Some laboratory and regulatory aspects of the control of *Cl. botulinum* in processed foods. In *"Botulism 1966"*. (Eds. Ingram, M. and Roberts, T.A.) p 511. London, Chapman and Hall Ltd.

Todd, E.C.D. (1997) Epidemiology of food borne diseases: a world wide review. *World health Statistics Quarterly*. 50, 30-50.

Troller, J.A. and Christian, J.H.B. (1978) *Water Activity and Food.* p. 131-173. London, Academic Press.

Trust, T.J. (1971) Bacterial counts of commercial fish diets. *J. Fish. Res. Bd. Canada.* 28, 1185-1189.

Tsang, N., Post, L.S. and Solberg, M. (1985) Growth and toxin production by *Clostridium botulinum* in model acidified systems. *J. Food Sci.* 50, 961-965.

Venkateswaran, K., Kiiyukia, C., Takaki, M., Nakana, H., Matsuda, H., Kawakami, H. and Hashimoto, H. (1989 b) Characterization of toxigenic vibrios isolated from the freshwater environment of Hiroshima, Japan. *Appl. Environ. Microbiol.* 55(10), 2613-2618.

Venkateswaran, K., Nakano, H., Okabe, T., Takayama, K., Matsuda, O. and Hashimoto, H. (1989 a) Occurrence and distribution of *Vibrio* spp. *Listeria* spp., and *Clostridium botulinum* in the Seto Inland sea of Japan. *Appl. Environ. Microbiol.* 55(3), 559-567.

Vijayakumaran, M. and Paulraj, R. (1997) Diseases of cultured penaeid shrimps in Nellore District, Andhra Pradesh. *J. Fish Dis.* (in press).

Ward B.Q., Carroll, B.J., Garrett, E.S., and Reese, G.B. (1967 b) Survey of the U.S. Atlantic coast and estuaries from Key Largo to Staten Island for the presence of *Clostridium botulinum*. *Appl. Microbiol.* 15(4), 964-965.

Ward, B.Q. and Carroll, B.J. (1965) Presence of *Clostridium botulinum* type E in estuarine waters of the Gulf of Mexico. *Appl. Microbiol.* 13, 502.

Ward, B.Q., Carroll, B.J., Garrett, E.S. and Reese, G.B. (1967 a) Survey of the U.S. Gulf coast for the presence of *Clostridium botulinum*. *Appl. Microbiol.* 15, 629-636.

Ward, B.Q., Garrett, E.S. and Reese, G.B. (1967 c) Further indications of *Clostridium botulinum* in Latin American Waters. *Appl. Microbiol.* 15(6) 1509.

Weber, J.T., Hibbs, R.G. Jr., Darwish, A., Mishu, B., Corwin, A.L., Rakha, M., Hatheway, C.L., Sharkawy, S.E., El Rahim, S.A., Al-Hamd, M.F.S., Sarn, J.E., Blake, P.A. and Tauxe, R.V. (1993) A massive outbreak of type E botulism associated with traditional salted fish in Cairo. *J. Infect. Dis.* 167, 451-454.

Wenzel, S., Bach, R. and Mûller-Prasuhn, G. (1971) Farmed trout as carriers of *Clostridium botulinum* and the cause of botulism. IV. Sources of contamination and contamination paths in fish farming and processing stations; ways to improve hygiene. *Archiv. Lebensmittel hyg.* 22, 131-135.

West, P.A. (1989) Human pathogens and Public health indicator organisms in shellfish. Chap. 12. In *Methods for the Microbiological examination of fish and shellfish.* (Eds. Austin, B. and Austin, D.A.) pp 273-308. Chichester, Halsted Press.

Wheaton, F.W. and Lawson, T.B. (1985) *Processing Aquatic Food Products.* New York, Inc, John Wiley and sons.

Wibowo, S., Putro, S., Suwarno, T., Sukarto and Wardoyo, S. (1991) Bacterial load of normal, moulting and delayed moulting farmed tiger shrimp (*Penaeus monodon fab*) *FAO Fish. Rep. 470 Supplement*, Rome, FAO. p 40-44.

Wignall, J., Mc Donald, I., Hill, G.P. and Macrae, M. 1990. Storage of iced gutted Haddock in carbon dioxide atmosphere. In *Processing and Quality of foods Vol.3 chilled foods. The revolution in Freshness* (Eds. Zenthen, P., Cheftel, J.C., Eriksson, C.,Germley, T.R., Linko, P. and Paulus, K.) pp. 3.204-3.210. London, Elsevier Applied Sciences

Wijewanta, E.A. (1962) Toxigenic strains of *Clostridium botulinum* in soils of *Ceylon*. *Ceylon J. Med. Sci.* 11, 59.

Williams-Walls, N.J. (1968) *Clostridium botulinum* type F: Isolation from crabs. *Science* 162, 375-376.

Willis, A.T. (1979) *Anaerobic bacteriology: Clinical and Laboratory Practice*. 3rd edition. London: Butterworth and Co. (Publishers) Ltd.

Willis, A.T. (1990) Clostridium the spore bearing anaerobes. In *Topley and Wilson's Principles of Bacteriology, Virology and Immunity* Vol. 2. *Systematic Bacteriology* (Eds. Tomparker, M. and Duerden, B.I.) pp. 212-246. London, Edward Arnold.

Wong, D.M., Young-Perkins, K.E. and Merson, R.L. (1988) Factors influencing *Clostridium botulinum* spore germination, outgrowth and toxin formation in acidified media. *Appl. Environ. Microbiol.* 54, 1446-1450.

Woodburn, M.J., Somers, E., Rodrigues, T. and Schantz, E.J. (1979) Heat inactivation rates of botulinum toxins A, B, E and F in some foods and buffers. *J. Food. Sci.* 44, 1658-1661.

Woolford, A.L., Schantz, E.J. and Woodburn, M.J. (1978) Heat inactivation of botulinum toxin type A in some convenience foods after frozen storage. *J. Food Sci.* 43, 622-624.

Yamamoto, K., Asano, H., Seito, V., Nakya, S., Horiuchi, Y., Awasa, K., Sasaki, J. and Kinura, K. (1970) Examination of *Clostridium botulinum* in samples taken from Lake Tourada. *Hirosaki Med.* J. 22, 92.

Yao, M.G., Denny, C.B. and Bohrer, C.W. 1973. Effect of frozen storage time on heat inactivation of *Clostridium botulinum* type E toxin. *Appl. Microbiol.* 25(3), 503-505.

Young-Perkins, K.F. and Merson, R.L. (1987) *Clostridium botulinum* spore germination, outgrowth and toxin production below 4.6: interactions between pH, total acidity and buffering capacity. *J. Food Sci.* 52, 1084-1088, 1096.

Zaleski, S.J. (1981) *Clostridium botulinum* type E, its occurrence and technological importance in the fish industry. In *Psychrotrophic microorganisms in spoilage and pathogenicity.* (Eds. Roberts, T.A., Hobbs, G., Christian, J.H.B. and Skovgaard, N.S.) p 485-489. London, Academic Press.